



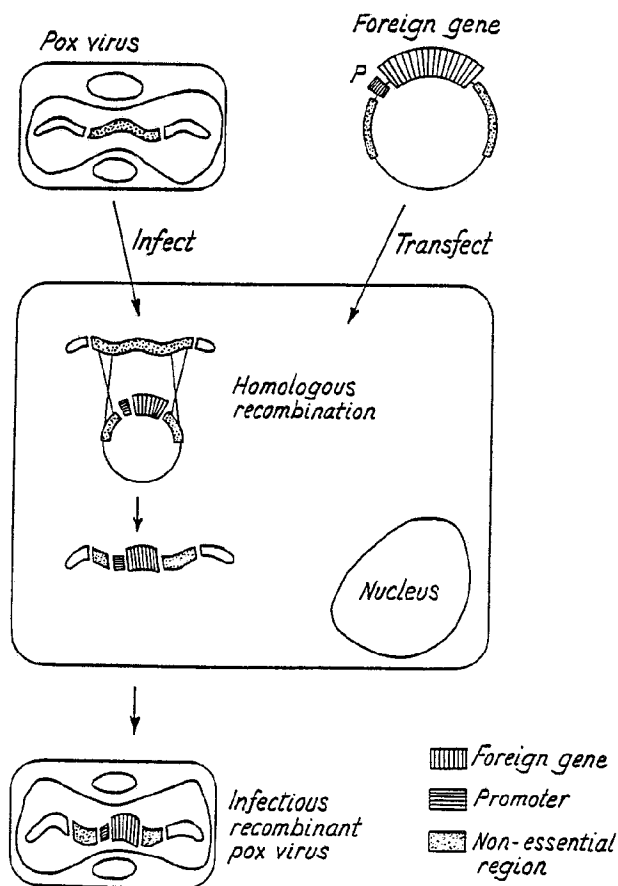
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: FOWLPOX VIRUS PROMOTERS

## (57) Abstract

Fowlpox virus (FPV) promoter DNA for use in expressing a foreign gene inserted in an FPV vector by homologous recombination, which comprises the promoter of any of the following FPV genes: (1) the FP4b gene which encodes a protein of about 657 amino acids in a sequence beginning Met Glu Ser Asp Ser Asn Ile Ala Ile Glu Glu Val Lys Tyr Pro Asn Ile Leu Leu Glu; (2) the *Bam*HI fragment ORF8 gene encoding a protein of about 116 amino acids in a sequence beginning Met Glu Glu Gly Lys Pro Arg Arg Ser Ser Ala Val Leu Trp Met Leu Ile Pro Cys Gly; (3) The *Bam*HI fragment ORF5 gene encoding a protein of about 105 amino acids in a sequence beginning Met Ile Ile Arg Arg Asn Asn Lys Ala Leu Gly Ser Val Met Ser Asp Phe Ile Lys Thr; (4) The *Bam*HI fragment ORF10 gene encoding a protein of about 280 amino acids in a sequence beginning Met Lys Phe Lys Glu Val Arg Asn Thr Ile Lys Lys Met Asn Ile Thr Asp Ile Lys Ile; and (5) the gene of which the coding strand hybridises strongly to FPV RNA and is at least partly located within an approximately 790 bp DNA sequence, containing near its 5'-end the sequence: (5') TGTCATCATA TCCACCTATA AATGTAA-TAT and near its 3'-end the sequence: AAGAATAGTC TAA-ATTACCT AACATAGAAC ATCAT (3').



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FOWLPOX VIRUS PROMOTERSBackground of the invention.1. Field of the invention.

The invention is in the field of recombinant DNA technology and relates to promoters useful for the expression of foreign DNA  
05 inserted into a fowlpox virus vector.

2. Description of the prior art.

Poxviruses are large viruses with a complex morphology containing linear double-stranded DNA genomes. They are among the few groups of DNA viruses that replicate within the cytoplasm  
10 of the cell. They are subclassified into six genera: orthopoxviruses, avipoxviruses, capripoxviruses, leporipoxviruses, parapoxviruses and entomopoxviruses. Vaccinia virus, an orthopoxvirus, is the most widely studied of the poxviruses, and is the subject of U.S. Patent 4,603,112 (Paoletti et al.).  
15 Fowlpox virus is an avipoxvirus or avian poxvirus.

Recent advances in recombinant DNA technology have allowed vaccinia virus to be used as a vector to carry and express foreign genes. For a review see M. Mackett & G.L. Smith, Journal of General Virology 67, 2067-2082 (1986). Certain properties of  
20 vaccinia virus make it suitable for this purpose. Firstly, it tolerates large amounts of extra DNA in its genome, at least up to 25,000 base pairs. Secondly, it encodes its own RNA polymerase which specifically initiates transcription of messenger RNA, beginning at the viral promoter sequences on the  
25 DNA genome. The host cell RNA polymerase II does not recognise these viral promoters, nor does the vaccinia RNA polymerase transcribe from promoters recognised by the host cell RNA polymerase. These two properties allow foreign genes to be inserted into the vaccinia virus genome under the control of a  
30 vaccinia virus promoter. Because of the very large size of the vaccinia virus genome (186,000 base pairs) and the fact that the DNA alone is not infectious, conventional recombinant DNA techniques of restriction enzyme cleavage and ligation of DNA fragments into the genome are not technically feasible.

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Therefore DNA is introduced into the genome by a process of homologous recombination. Homologous recombination involves essentially (1) pre-selecting a length of the vaccinia virus (VV) genome in some region which does not impair the replication and normal functioning of the virus (hereinafter called a "non-essential region"), (2) making a construct of a length of foreign DNA in a copy of the non-essential region so that the foreign DNA is flanked by extensive sequences of non-essential region of VV DNA, (3) co-infecting appropriate tissue culture cells with the VV and with the construct and (4) selecting cells containing VV in which the pre-selected length has been swapped over ("recombined") in vivo so that it is replaced in the genome by the construct DNA.

In order to insert the foreign gene into the construct, the construct should itself be contained in a vector, e.g. a plasmid. It should also comprise a promoter for regulating expression of the foreign DNA within the virus. The procedure is more fully described in the Mackett and Smith review supra. Vaccinia virus vectors have been used in this way experimentally for the expression of DNA for several viral proteins. See, for example, M. Kieny et al., Nature 312, 163-166 (1984) on the expression of a rabies virus glycoprotein. Since the vaccinia virus vector can be attenuated, i.e. altered to make it less virulent, without impairing its use as a vector, it has considerable potential for use in vaccination.

It has been recognised for some years that in principle similar technology could be applied to fowlpox virus (FPV), see, for example, M.M. Binns et al., Israel Journal of Veterinary Medicine 42, 124-127 (1986), thereby providing a vector for use in vaccinating poultry. FPV like VV, has a genome of vast size (it is even larger than VV: estimates range from 240 to 360 kilobases) and it is not known to what extent it is similar to vaccinia virus.

One of the essential requirements for the expression of foreign DNA in a FPV vector is a strong promoter, which will be

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recognised by the FPV RNA polymerase. Several promoters have been identified in VV but their relative strengths have not been fully explored. The main ones are as follows:

- 05 1. p7.5. The 7.5 Kd polypeptide promoter, which has early and late activities, has been widely used to express genes inserted into vaccinia, S. Venkatesan et al., Cell 125, 805-813 (1981), M.A. Cochran et al., J. Virol. 54, 30-37 (1985).
- 10 2. p11. The gene for the 11 Kd major structural polypeptide, mapping at junction of vaccinia HindIII fragments F/E, has a late promoter which has been widely used, C. Bertholet et al. Proc. Natl. Acad. Sci. USA 82, 2096-2100, (1985).
- 15 3. pTK. Promotes the thymidine kinase, gene which maps in vaccinia HindIII fragment J, J.P. Weir et al., Virology 158 206-210 (1987). This promoter has not been used much and is thought not to be strong.
4. pF. Promotes an unknown, early, non-essential gene, which maps in vaccinia HindIII fragment F, see D. Panicali et al. Proc. Natl. Acad. Sci. USA 80, 5364-5368 (1983). It has recently shown to be "relatively inefficient" i.e. 10-fold lower than the
- 20 TK promoter, B.E.H. Coupar et al., J. Gen. Virol. 68, 2299-2309 (1987).
5. p4b. The 4b gene encodes a 62 Kd core protein. It has a late promoter which maps in vaccinia HindIII fragment A, see J. Rosel et al., J. Virol. 56, 830-838 (1985). The 4b protein accounts
- 25 for approx 10% of viral protein in vaccinia.
- 6 and 7. pM. and pI. These are two uncharacterised early vaccinia promoters from vaccinia HindIII M and I fragments respectively used in construction of a multivalent vaccinia vaccine, M.E. Perkus et al., Science 229, 981-984 (1985).
- 30 8. p28K. Promotes a gene encoding a later 28 Kd core protein, J.P. Weir et al., J. Virol. 61, 75-80 (1987). It hasn't been used much.

Because of the lack of information about the genomic DNA sequence of FPV (and, indeed, VV, since only about a third of the

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genomic DNA sequence of VV has been published), it has not been possible to predict whether a particular promoter known in VV has a counterpart in FPV, nor could its efficiency as a promoter be predicted.

05        Only very limited data have been published about the DNA sequence of the FPV genome. Thus, D.B. Boyle et al., Virology 156, 355-365 (1987), have published the sequence of the thymidine kinase (TK) gene and flanking sequence totalling 1061 base pairs. These authors looked at the FPV TK promoter region and  
10        noted that it contained a so-called consensus sequence common to eleven VV gene promoters [A. Plucienniczak et al., Nucleic Acids Research 13, 985-998 (1985)]. This "consensus sequence" is supposedly based on TATA --- (20 to 24 bp) -- AATAA, but there were many divergences from it and the whole region is so AT-rich  
15        that the notion of a "consensus sequence" does not bear critical examination. Moreover, the distances between these consensus sequences and the 5' ends of the TK mRNAs differed as between FPV and VV. Since the FPV TK gene was found to be expressed in vaccinia virus vector, and therefore recognised by the VV RNA  
20        polymerase, some degree of similarity between these two promoters is deducible. It does not follow, of course, that every VV promoter would be highly homologous with every FPV promoter and indeed unpublished data of the present inventors suggests that this is not the case.

25        Further prior art is referred to below after the section "Summary of the invention", without which its context would not be apparent.

#### Summary of the invention

30        Much of the present invention has arisen by locating some FPV genes, testing the 5'-non-coding region associated with them for promotional strength and thereby selecting certain strong promoters.

Several regions of the FPV genome have been investigated in research leading to the invention. One of them arises by cutting

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the DNA with the enzyme BamHI, selecting from a range of plasmids thereby generated one with an insert of about 11.2 kilobases and examining that length of DNA. Another arose by random-cloning of the FPV genome and comparing these sequences with that of DNA of the vaccinia 4b gene mentioned above.

Another method of identifying strong promoters involved simulating the transcription of RNA from the FPV DNA.

As a result, five strong promoters have been found and the invention provides various DNA molecules containing them. The science of promoters of poxvirus DNA is at present poorly understood. It is known that certain regions to the 5' or "upstream" end of a gene serve to assist in transcribing genomic DNA into messenger RNA by binding the RNA polymerase involved in the transcription so that the mRNA which contains the start codon of the gene can be transcribed. Such upstream regions are referred to as the "promoter". It is often not possible to say for certain which nucleotides of the upstream sequence are essential and which are inessential for promotion, nor is the minimum or maximum length of the promoter known with great precision. Although this lack of precision in the whereabouts and length of the promoter might at first sight seem rather unsatisfactory, it is not a problem in practice, since there is normally no harm in including additional DNA beyond the region which serves to transcribe the DNA. Further as described later, it is possible by tedious experiment to determine this region more precisely. In all these circumstances, it is therefore more appropriate to define the promoter by reference to the gene which it precedes, rather than by reference to the sequence of the promoter. Four of the genes in question are those of open-reading frames ORF8, ORF5 and ORF10 of the BamHI fragment and the gene of FPV which most nearly corresponds to (is of highest homology with) the vaccinia 4b gene. The last-mentioned FPV gene is conveniently designated FP4b. These genes are fully identified hereinafter in Example 1.

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The fifth strongly promoted gene was identified by research into amounts of mRNA likely to be produced when viral DNA is transcribed. The theory is that strong promoters direct the transcription of greater amounts of RNA than weak promoters. In order to avoid the problems of experimentation in vivo, RNA was prepared in vitro in a manner thought likely to emulate in vivo transcription. The RNA thus prepared was hybridised to BamHI and EcoRI restriction fragments of FPV DNA. Strong hybridisation to several fragments was taken to indicate a strongly promoted gene and by this means such a gene which falls at least partly within a 0.79kb EcoRI fragment was identified and the fragment partly sequenced: see Example 2.

These five genes can be defined in various ways, always remembering, of course, that there will doubtless be minor differences in their sequence between one strain or type of FPV and another. One convenient, arbitrary, way of defining them is by reference to an appropriate length of the amino acid sequence which they encode. It may reasonably be assumed that the first 10 or, more preferably, the first 20 amino acids, say, would form a unique sequence in FPV. Accordingly, one convenient definition of four of the genes is based on the first 20 amino acids as follows :-

- (1) The FP4b gene which encodes a protein of about 657 amino acids in a sequence beginning
 

Met Glu Ser Asp Ser Asn Ile Ala Ile Glu  
 Glu Val Lys Tyr Pro Asn Ile Leu Leu Glu
- (2) The BamHI fragment ORF8 gene encoding a protein of about 116 amino acids in a sequence beginning
 

Met Glu Glu Gly Lys Pro Arg Arg Ser Ser  
 Ala Val Leu Trp Met Leu Ile Pro Cys Gly
- (3) The BamHI fragment ORF5 gene encoding a protein of about 105 amino acids in a sequence beginning
 

Met Ile Ile Arg Arg Asn Asn Lys Ala Leu  
 Gly Ser Val Met Ser Asp Phe Ile Lys Thr



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(4) The BamHI fragment ORF10 gene encoding a protein of about 280 amino acids in a sequence beginning

Met Lys Phe Lys Glu Val Arg Asn Thr Ile

Lys Lys Met Asn Ile Thr Asp Ile Lys Ile

05 - Gene (5) could be defined as at least partly located within a 790bp (0.79kb) DNA sequence, containing near its 5'-end the sequence:

(5') TGTCATCATA TCCACCTATA AATGTAATAT and near its 3'-end the sequence:

10 AAGAATAGTC TAAATTACCT AACATAGAAC ATCAT (3')

In relation to genes (1) to (4), it will be appreciated, of course, that variations in the 20 amino acids are likely to occur between different FPV strains. Probably there would be at least 90% homology over the whole gene, but there may well be less  
15 homology over the first 20 amino acids, perhaps up to 3 or 4 differences. It is confidently believed however that no one skilled in the field will be in any doubt as to which gene is intended, whatever the precise degree of aberration in the amino acid sequence of the first 10 or 20.

20 Likewise, in relation to gene (5) it will be appreciated that the quoted DNA sequence is that of the EcoRI fragment detected, that in some strains of FPV one or both of the EcoRI restriction sites might be lacking and that consequently it is more definitive to quote the DNA sequence. With the aid of the  
25 sequence information given herein for the 790 bp fragment it will readily be possible to complete the sequencing of the 790 bp DNA and then find an open-reading frame (ORF) for gene (5). This gene does not necessarily fall wholly within the 790 bp fragment. Thus, it might be necessary to sequence the genome to  
30 either side of the 790 bp region. This could be done by labelling the 790 bp DNA and using it to probe a library of the FPV genomic DNA made by restriction with a different enzyme. When the beginning of the ORF is located, the 5'-non-coding sequence can be used as promoter DNA. If there should perchance

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be two genes falling within this fragment, whichever hybridises more strongly to the RNA is intended. Although there might be some nucleotide variation between strains of FPV, there would probably be at least 80% homology at the DNA level. It is  
05 confidently believed, however, that no one skilled in the art will be in any doubt as to which gene is intended.

It is expected that before long it will be possible to create a partial map of the FPV genome. FPV, like other poxviruses, has a linear genome with similarities between its ends: The terminal  
10 sequences are invertedly repeated. Within these terminal inverted repeats (TIRs) there are tandemly repeated sequences. The BamHI digest gave rise to clones containing these terminal inverted repeat (TIR) sequences and it has been determined that a length of about 3.7 to 4.0 kb at one end of the approximately  
15 11.2 kb fragment (the left-hand of the sequence thereof shown hereinafter) lies within a TIR in the strain of FPV investigated. The FP4b gene is believed to lie in a central region of the genome. The whereabouts of the 0.79 kb sequence is unknown at present.

20 The invention includes a DNA molecule which consists substantially of the non-coding DNA to the 5'-end of each of the above-identified genes and comprising the promoter thereof. "Non-coding" means not coding for that gene : it could code for another gene as well as serving as a promoter. Any reasonable  
25 length of such DNA, typically up to 150, usually up to 100, and especially up to 80 nucleotides (or base-pairs in the case of ds DNA) of the 5'-end (even if it codes for DNA within the next gene along the genome), is herein referred to as "promoter DNA".

The invention also includes a recombination vector comprising  
30 a cloning vector containing a non-essential region (NER) sequence of FPV, said NER being interrupted by DNA which consists of or includes (a) promoter DNA of the invention, followed by (b) a foreign gene (i.e. a gene which it is desired to insert into the FPV vector) transcribable by the promoter.

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In one particular aspect, the invention includes a recombination vector which comprises in order :

- (1) a first homologously recombinable sequence of the fowlpox virus (FPV) genome,
- 05 (2) a sequence within a first portion of a non-essential region (NER) of the FPV genome,
- (3) FPV promoter DNA according to the invention,
- (4) a foreign gene transcribably downstream of the promoter (whereby when the fowlpox virus RNA polymerase binds to the
- 10 promoter it will transcribe the foreign gene into mRNA) and
- (5) a sequence within a second portion of the same NER of the FPV genome, the first and second sequences preferably being in the same relative orientation as are the first and second portions of the NER within the FPV genome, and
- 15 (6) a second homologously recombinable sequence of the FPV genome, said sequences (1) and (6) flanking the NER in the FPV genome and being in the same relative orientation in the recombination vector as they are within the FPV genome.

In another aspect, the invention includes a DNA construct  
20 which comprises a promoter of the invention transcribably linked to a foreign gene. Such a construct or "cassette" can be inserted in a cloning vector, which can then be used as a recombinant vector useful in preparing a recombination vector of the invention.

25 The invention further includes hosts harbouring the recombination and recombinant vectors of the invention, especially a bacterial host harbouring a plasmid vector.

The invention is further directed to a recombinant FPV which is the product of homologous recombination of FPV with a  
30 recombination vector of the invention containing a foreign gene; the process of homologous recombination; animal cells infected with such a recombinant FPV; a process of in vitro culture of these infected cells; and a method of vaccinating a responsive animal, especially a chicken, which comprises inoculating it with  
35 the recombination vector of the invention.

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Further description of the prior art

At the International Poxvirus Workshop meeting held at Cold Spring Harbor, New York, on 24-28 September 1986, F.M. Tomley gave a talk, with slides, entitled "Molecular structure and organisation of an 11.3 kb fragment of fowlpoxvirus". This talk presented an outline of the preliminary results of sequencing the 11.2 kb BamHI fragment (at that time thought to be 11.3, rather than 11.2 kb long). The talk dealt with the AT richness of the fragment, included a slide showing 20 open reading frames, discussed codon usage in FPV, compared the FPV 48 kd predicted polypeptide (herein "ORF 1") with a 42 kd early protein in VV and compared other predicted polypeptides with hepatic lectins and anti-alpha-trypsinogen. No mention was made of the functionality of the ORFs or of the strength of gene expression, nor was any length of DNA sequence shown. The same talk was given at the Herpes/Poxvirus Workshop of the Society for General Microbiology, held at St. Andrews, Scotland, April 1987.

At the corresponding meeting in September 1987, J.I.A. Campbell et al., displayed a poster relating the terminal BamHI fragment of FPV, lying between the 11.2 kb BamHI fragment and the end of the genome. No DNA sequence was shown.

During the priority year, F.M. Tomley et al., J. Gen. Virology 69, 1025-1040 (1988), have given the full sequence of the BamHI fragment, together with some detail of relationships of predicted polypeptides to other proteins. A study of the functional promoter activity of the sequences upstream of the 12 major ORFs is referred to as unpublished data. The first disclosure of this data was in a poster exhibited by M.E.G. Bournsnel et al., at the VIIth International Poxvirus/Iridovirus Meeting, Heidelberg, 22-26 August 1988.

Brief description of the accompanying drawings

Fig. 1 shows the general scheme of a procedure of homologous recombination as applied to fowlpox virus;

Figure 2 and 3 are plasmid maps showing schematically the derivation of recombination vectors of the invention useful in

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the homologous recombination; and

Fig. 4 is a plasmid map showing the derivation of a construct for testing FPV promoters of the invention in a transient assay.

Description of the preferred embodiments

05 While the precise length of DNA required for promotion is not known, it is generally reckoned to be up to 100 base pairs from the RNA start site, but this can be as much as 50 base pairs away from the gene start site (the ATG codon). Accordingly a DNA sequence contained within 150 base pairs, less preferably 100 or  
10 even 80 bp, to the 5'-end of the gene (immediately preceding the start codon) is of particular interest for the purposes of the invention. The DNA sequences of these 150 base pairs are shown below (arbitarily divided into blocks of 10 for ease of reading) for genes (1) to (4).

FP4b (5') TATTACGTGG ATAAATATAT ATCTTCAGGA AAAGGGTATT ATGTTACCAG  
ATGATATAAG AGAACTCAGA GATGCTATTA TTCCTTAACT AGTTACGTCT  
CTTTAGGTAC TTATTTTGAT ACGTTACAAG TAAAAAACTA TCAAATATAA  
(3')

ORF8 (5') AGAATAGCAT TGCAAAGTTC TACACGATCC ATTGTATAAT ATAGGTGTTC  
AACACCTCTC GATATATCAT TATTTGTTTT TTCAATTTTA TTATAAGTAG  
TTTGAATGCA TTTTAAAGTT TAATAAATCT TGATAAAGTA TATTTAAAAA  
(3')

ORF5 (5') TAAACCAAAT ATACTAAAAT ATAAAATTAT GCCGCGGGAT GATAAGATAC  
TTCAGATGAT CGTGATGAAC TATATTTATT AATTGGCAAT ACTTAAAAAT  
AATGTTTATA ACATATGTAA ATATAATAAA CAATAATTTA GATTTTAAAA  
(3')

ORF10 (5') ACTAGATTGT ACAAATATTA ATATGTGTAA TTTCTTATAT AGTAATATAG  
TAGGATGTGA TATATGCACC ATAGAAAAAT TTTATATTTG TATAAAACCG  
ATAAATAAAA TAAACTTATT TAGTTACTTT GTAGAGTATA CTAAATAATA  
(3')

15 In the above sequences an ATG start codon follows on at the right-hand or 3'-end.

Just how much of the 5'-non-coding sequence is necessary for efficient promotion is not known precisely. However, experiments

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can be carried out to answer this question, and in fact some have been performed for VV. Consequently, similar experimentation would be possible to determine the sequences necessary for FPV. One such technique is deletion mapping: by the simple expedient of removing parts of the sequence under test, and assaying its subsequent promotion efficiency, the sequences sufficient for promoter activity can be identified. Thus, in vaccinia it has been found that 100 base pairs (bp) of sequence upstream of the 11 kilodalton (11Kd) gene are sufficient to act as a promoter and temporally regulate late transcription C. Bertholet *et al.*, Proc. Natl. Acad. Sci. (USA) 82 2096-2100 (1985). Deletions leaving about 15 bp on the 5'-side of the putative site at which mRNA transcription starts still yielded high levels of expression, C. Bertholet *et al.*, EMBO Journal 5, 1951-1957 (1986). However, M Hanggi *et al.*, EMBO Journal 5 1071-1076 (1986) found that the same fragment functioned at a lower level when it was translocated to a new position. At this new position, deletions leaving 32 bp on the 5'-side of the ATG start codon had no effect on promoter strength. M.A. Cochran *et al.*, Proc. Natl. Acad. Sci (USA) 82, 19-23 (1985) showed that the activity of the 7.5Kd VV promoter resided in an approximately 30 bp segment. J.P. Weir and B. Moss, Virology 158, 206-210 (1987) found that 32 bp upstream of the RNA start site were sufficient for correctly regulated promotion of the thymidine kinase (TK) gene in VV.

A 228 bp sequence of DNA from in front of a 28Kd late gene (from positions -218 to +10 relative to the RNA start site) was placed in front of the chloramphenicol acetyltransferase (CAT) gene and found to act as a promoter, J.P. Weir and B. Moss, J. Virology 61, 75-80 (1987). A series of 5' deletions extending towards the RNA start site were made. A gradual reduction in CAT expression occurred as the deletions extended from -61 to -18. Mutants that retained 18 bp before and 10 bp after the RNA start site still expressed the CAT gene as a late gene, though at a submaximal level.

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While deletion mapping can define those sequences sufficient for promotion activity, it cannot pinpoint the exact bases necessary for activity within the defined sequences. Various workers have altered bases within putative promoter sequences, either by synthesising specific oligonucleotides, M. Hanggi et al., loc. cit., or by site-directed mutagenesis, J.P. Weir and B. Moss, J. Virology 61, 75-80 (1987). In both cases alterations in very few, even single, bases had profound effects on the efficiency of promotion, and hence individual bases of importance could be identified. Since, however, some changes in sequence are permissible without loss of the promotional effect, it will be appreciated that it is necessary that the invention should cover sequences which are variant, by substitution as well as by deletion or addition from the non-coding sequences of length up to 150 bp referred to above.

The recombination vector could contain additional sequence to that herein referred to as promoter DNA. Additional sequence could comprise (a) additional sequence more than 150 bp 5'-ward from the ATG initiation codon, (b) sequence inserted into the 150 bp without destroying promoter activity or (c) part of the sequence of the FPV gene (inclusive of the ATG initiation codon and onwards), e.g. up to 100 bp thereof.

The above experiments require testing for the efficiency of the promoter. It is not necessary for this purpose to introduce a promoter-gene construct into FPV and monitor expression of the gene product. A shorter method, known as transient assay, is known for use with VV, M.A. Cochran et al., Proc. Natl. Acad. Sci. (USA) 82, 19-23 (1985). In transient assay, the promoter is linked to a gene with an easily assayable product. A plasmid containing this construct is then introduced into a cell which has been infected with the virus. The viral RNA polymerase can transcribe off the promoter, even though the promoter has not been incorporated in the viral genome. Because expression only

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lasts while both the virus and the plasmid DNA are present in the cell together, this form of expression is known as 'transient'. Two different marker genes have been used in vaccinia virus transient assay systems, the chloramphenicol acetyltransferase (CAT) gene, M.A. Cochran *et al.*, *supra* and the beta-galactosidase "lacZ" gene, D. Panicali *et al.*, Gene 47 193-199 (1986). Using the CAT gene the promoter sequences under test were cloned in front of a complete CAT gene which included its own ATG start codon. Thus, this is a "transcriptional fusion" sequence, i.e. the sequences are fused in a non-coding region. In the case of the beta-galactosidase lacZ gene both a transcriptional and a translational fusion vector were described, both for transient assay and for testing in recombinants. The translational fusion vector contained a beta-galactosidase gene lacking its own start codon, so that the fusion occurs within a coding region. The ATG start codon was provided by the VV promoter under test. The beta-galactosidase "lacZ" gene was therefore cloned so as to be in frame with the VV gene start codon, the VV gene being fused to the lacZ gene before codon 9 of the latter. Thus, the promoter was in exactly the same context relative to the initiation codon used in the fusion vector as in its native position.

In the present invention, the lacZ gene has been used only for the transient assay to determine promoter strength. It will be appreciated, however, that in the practice of the invention a foreign gene relevant to improving the condition of poultry would be inserted into the fowlpox virus. Preferably the gene will be one appropriate to an in vivo sub-unit vaccine, for example one or more genes selected from Infectious Bronchitis Virus (IBV), Infectious Bursal Disease virus, Newcastle Disease Virus (NDV), Marek's disease virus, infectious laryngotracheitis virus and genes encoding antigenic proteins of Eimeria species. Particular genes of interest are the spike genes of IBV and the HN and F genes of NDV as described in PCT Patent Application Publication No. WO 86/05806 and European Patent Application Publication No. 227414A (both National Research Development Corporation). In



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order for the foreign gene to be correctly translated in vivo it is necessary for the foreign gene to have its own ATG start codon inserted in the region just following the promoter.

It is necessary to locate a non-essential region of the FPV, in which to insert the promoter of the invention and the desired foreign gene. In principle, they could be inserted anywhere in the FPV genome which would not harm the basic functions of the virus, or interfere with the action of the FPV promoter or the foreign gene. It can be a coding or non-coding region. In VV, the thymidine kinase (TK) gene has often been used for this purpose. See, for instance, Example 4 of WO 86/05806 mentioned above, which describes the expression of the IBV spike gene in VV using the 7.5K vaccinia promoter and the TK non-essential region.

It will be appreciated that the detection of the insertion of the foreign gene would depend on detection of virally infected cells which do not produce any of the non-essential gene, e.g. TK. Such cells are described as "TK minus". Alternatively, one could use the TK gene or a markerless coding or non-coding region and detect the insertion of the foreign gene by a hybridisation assay in which a labelled nucleotide sequence complementary to the foreign gene sequence is employed.

PCT Application WO 88/02022 published 24th March 1988 (CSIRO) describes a method of stably inserting a foreign gene within the TK gene of FPV, with the aid of a dominant selectable marker gene ("Ecogpt") and a VV promoter. The disclosure of this patent application can be used in the present invention, with substitution of an FPV promoter of the invention for the VV promoter. Use of the FPV promoter is favoured as likely to be more acceptable to the veterinary medicine licensing authorities.

The promoter of the invention and foreign gene then have to be inserted into the non-essential region (NER) of the FPV genome. The procedure of homologous recombination illustrated by Figure 1 of the drawings, provides a way of doing so. A fragment of genomic DNA containing the NER is sub-cloned in a cloning vector. If desired, it can be shortened to remove most of the

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sequence flanking it. A construct is then made, in the cloning vector, comprising part of the NER (starting at one end thereof), followed by the FPV promoter ("P") of the invention, followed by the foreign gene, followed by substantially the remainder of the NER (terminating at the other end thereof). This construct, in an appropriate vector, forms the recombination vector which is used to transfect the cells infected with the FPV, e.g. by the calcium phosphate method, whereby recombination occurs between the NER sequences in the vector and the NER sequences in the FPV. The FPV then automatically re-packages this altered genome and the thus altered FPV (recombinant FPV) is part of this invention.

Figures 2 and 3 of the drawings illustrate alternative methods of making the above recombination vector. Referring first to Figure 2, a non-essential region possessing two restriction sites A, B is inserted in an appropriate vector, which, by way of illustration only, will be described as a plasmid. In another plasmid having the same (or ligatably compatible) restriction sites A, B, a construct is made of FPV promoter sequence of the invention followed by the foreign gene sequence. It is of course essential that this construct is made so that the mRNA transcription will begin at or before the start codon of the foreign gene. Since it is time-consuming to determine precisely where the mRNA transcription start is effected by any particular promoter, it is convenient simply to insert, say, 100 or more preferably 150 base pairs of promoter DNA immediately preceding the FPV gene which it normally promotes, to ensure good working of the promoter. However, it will be appreciated that, given the time to do experiments previously indicated, portions of promoter DNA could be "chewed off" by restriction enzyme treatment to shorten it, thereby eliminating any unnecessary sequences. Such adaptation is considered to be an immaterial variation of the particular embodiments of the invention described herein. Equally, it would

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be possible to extend the promoter sequence at the downstream end thereof, e.g. to include a few base pairs of its natural FPV gene sequence. This would normally result in expression in vivo of a translational fusion protein if the foreign gene sequence is arranged to be in frame with the natural FPV gene. However, such a protein is not particularly desired and in fact any short sequence of nucleotides could be positioned between the promoter DNA and the start codon of the foreign gene.

The restriction sites A, B are located in the plasmid DNA flanking the FPV promoter DNA and the foreign gene. Of course, A could be within the promoter DNA if it falls within a non-functional portion thereof. While two different restriction sites have been shown for simplicity they could of course be the same. They can be sticky- or blunt-ended sites and can be prepared artificially by filling in and/or ligating additional nucleotides, in ways well known in the recombinant DNA field. Conveniently A and B in the type 2 construct are converted into identical blunt-ended sites (C, not shown) and then allowed to recombine at a single blunt-ended site C (replacing A, B) within the NER. Care will have to be taken, of course, to select sites which are unique in the vector DNA to prevent recombination of other sequences of DNA from occurring.

DNA from the two plasmids are ligated together in vitro and then transformed into the host, with suitable restriction enzymes, to produce the final construct of type 1. The promoter-foreign gene construct of type 2 is, of course, made in a similar way from a vector containing the promoter and another containing the foreign gene.

Figure 3 illustrates another method of preparing recombinant vectors of the invention. In this method one first prepares a construct comprising a first part of the NER followed by the FPV promoter of the invention, followed by a short sequence of nucleotides containing at least one cloning site for introduction of a foreign gene, followed by a second part of the NER, which

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could be simply substantially the remainder of the NER. Of course, virtually any length of DNA would provide a cloning site suitable in some way or other for introducing a foreign gene. Preferably these constructs contain a multiple cloning site, that  
05 is to say a short length of DNA containing the sites of a variety of different restriction enzymes, for example at least ten. Such a construct then has versatility, since it will then be much easier to restrict DNA flanking almost any foreign gene at sites close to each end thereof and insert the foreign gene into the  
10 multiple cloning site illustrated in Figure 3. Only two sites X, Y have been shown, for simplicity and, again, these can be filled in and extended or chewed back, as desired, to give identical blunt-ended sites (Z, replacing X, Y). In the final constructs, the promoter DNA will be separated from the foreign gene by a  
15 portion of the multiple cloning site, but this will not adversely affect the transcription of the mRNA in the final virus.

In either method of construction, the NER is split by the promoter and foreign gene. It is, of course, not essential that it be split in a central region. Nor is it essential that the  
20 second portion of the NER constitute the entire balance or remainder of the NER. So long as each end of the NER contains or is flanked by a long enough stretch of DNA for homologous recombination, it does not matter that a part of the NER might be excised somewhere in between or that additional (irrelevant) DNA  
25 be inserted in preparing the recombination vector. Obviously, it is not necessary that the NER used be the complete region or gene identified in the FPV genome as non-essential. Any part of it will do, and the term "end" in relation to the NER then means the end of the selected part.

30 References herein to vectors other than FPV (or VV) mean any convenient prokaryotic or eukaryotic cloning vector appropriate for bulk production of the construct within a suitable host. Prokaryotic vectors will ordinarily be plasmids or phages. Suitable prokaryotic hosts include bacteria. Eukaryotic vectors

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such as those of fungi, yeasts and animal cells, e.g. SV40, can be employed if thought more convenient.

Although the recombination vector used will ordinarily be of double-stranded DNA, it is possible to use single-stranded DNA  
05 for the homologous recombination.

The recombination plasmid of the invention containing the NER, promoter and foreign gene then has to be "swapped over" for FPV DNA in a homologous recombination procedure. For this purpose, appropriate poultry cells are infected with FPV. It is  
10 best not to use wild type FPV for obvious reasons. FPV can readily be attenuated (mutated to make it less virulent), by any conventional method of attenuation.

Many different methods are available for selecting the recombinant viruses, and have been described for VV in the review  
15 article of M. Mackett and G.L. Smith supra. Such methods are applicable in the present invention. Using the TK gene as the NER, one method is to transfer the mixture of viruses containing the desired (recombinant) virus to fresh TK minus cells in a growth medium containing BUdR. BUdR kills the original virus  
20 which was TK positive, so the TK minus mutants produced according to the invention can be selected. Another method is to enlarge the recombination plasmid to include a FPV or, less desirably, a VV promoter together with an additional marker gene, preferably selectable such as Ecogpt, but possibly non-selectable such as  
25 beta-galactosidase, within the NER and then detect recombinants by using a property of the marker gene, e.g. for beta-galactosidase the blue plaques generated when the 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal) substrate is present in the growth medium.

30 The selected TK minus cells containing the FPV (which has a deleted TK gene but possesses the foreign gene) are then grown in chicken embryo fibroblasts (CEFs), chicken fibroblasts, chick embryo epithelial cells derived by conventional tissue culture methods, principally trypsinisation of tissues or the

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chorioallantoic membrane (CAM) of embryonated chicken or turkey eggs. For administration to birds, the recombinant virus can be given to birds by aerosol, drinking water, oral, intramuscular injection or inoculation into the wing web. Ingredients such as  
05 skimmed milk or glycerol can be used to stabilise the virus.

While the invention is intended primarily for the treatment of chickens it is potentially of interest in relation to other animals which might safely be infected with FPV. It is even possible that it might be considered safe to infect humans with  
10 FPV after appropriate trials have taken place.

The following Examples illustrate the invention.

#### EXAMPLE 1

##### MATERIALS AND METHODS.

###### 1. Virus strain.

15 The HP438 strain of fowlpox virus was obtained from Professors A. Mayr and H. Mahnel, Ludwig-Maximilians University, Munich. The HP438 strain has been obtained from the pathogenic HP1 strain by 438 passages in chick embryo fibroblasts (CEFs) in tissue culture A. Mayr et al., Zentralblatt für Veterinärmedizin  
20 B13, 1-13 (1966). The HP441 strain used to obtain DNA for cloning was derived by 3 further passages in CEF cells.

###### 2. Tissue culture medium.

CEF cells were grown in 199 (Wellcome) medium, supplemented with Penicillin (200U/ml, Streptomycin (200µg/ml, Fungizone  
25 (2µg/ml) and 10% newborn calf serum (CS).

###### 3. Purification of virus and extraction of DNA therefrom.

HP441 fowlpox virus was inoculated on to confluent monolayers of CEF cells at a multiplicity of infection of approximately 1 plaque forming unit (pfu) per cell. Cells were pre-washed in  
30 serum-free medium, and the virus inoculum was added to the cells in 1ml of serum-free medium per 75cm<sup>2</sup> bottle. After 10 minutes incubation at 37°C to allow the virus to adsorb to the cells, 10ml of medium containing 2% calf serum (CS) was added. After 5 days, a marked cytopathic effect (CPE) was observed, at which

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time the supernatant was collected. Cellular debris was removed from the supernatant by centrifuging at 2500 rpm for 10 minutes in a Sorvall GSA rotor. The virus was then pelleted from the supernatant by centrifugation at 14000 rpm for 30 minutes in an  
05 Sorvall SS34 rotor. The viral pellet was resuspended in 10mM Tris pH9.0 and a further low speed spin performed to remove any remaining cellular material.

To extract the DNA from the virus, an equal volume of lysis buffer (100mM TRIS-HCl pH 7.8, 2mM EDTA, 54% sucrose, 2% SDS,  
10 200mM 2-mercaptoethanol) was added to the virus suspension. Proteinase K was then added as a solid to 500µg/ml. This was incubated at 50°C for 2 hours and then overnight at 4°C. The solution was then extracted slowly and gently for several hours with phenol/- chloroform/isoamyl alcohol (50:48:2 v/v/v,  
15 saturated with 10mM TRIS-HCl pH 7.5, 1mM EDTA) and then with ether. 2.5 volumes of absolute ethanol were added to precipitate the viral DNA. Viral DNA was resuspended in 10mM TRIS-HCl pH7.5, 1mM EDTA (TE) or in deionised water.

#### 4. Cloning of viral DNA into plasmid vectors.

20 1µg of FPV DNA was cut with the restriction enzyme BamHI (BRL) and ligated into BamHI-cut, phosphatase-treated pUC13 plasmid (Pharmacia). Following transformation into E. coli strain TG1 using standard methods, D. Hanahan, J. Mol. Biol. 166, 557-580 (1983), colonies containing plasmids with inserted DNA  
25 fragments were identified by a white colour on X-gal indicator plates. Colonies were probed with nick-translated (radio-labelled) FPV DNA and plasmids containing FPV DNA inserts were analysed by restriction digests of plasmid DNA isolated by the method of D.S. Holmes et al., Anal. Biochem. 114, 193-197  
30 (1981) and also of DNA purified on CsCl gradients. A range of recombinant plasmids containing FPV DNA inserts was obtained, and one of these, called pMH23, of approximately 11.2 kilobases, was selected for sequencing. EcoRI clones of FPV DNA were made in the same way, except that colonies were not probed with

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radiolabelled viral DNA but were stored in glycerol cultures as a 'library'.

5. Sequencing of pMH23.

05 To sequence the viral insert of pMH23, random subclones of pMH23 were generated by cloning sonicated fragments of pMH23 into SmaI-cut, phosphatase-treated M13mp10 (Amersham International PLC). Clones containing viral inserts were identified by colony hybridisation with radiolabelled insert from pMH23. Dideoxy sequencing with [<sup>35</sup>S]dATP was used to determine the complete  
10 sequence of the viral insert.

6. Random sequencing of the fowlpox virus genome.

Recombinant plasmids containing fowlpox DNA inserts were obtained by a similar method to the above, but starting from virus passaged a further three times (HP444). Random sequencing  
15 of the viral genome was carried out as in section 5 above. Sonicated fragments of viral DNA were cloned into M13mp10 and sequenced directly without any identification step.

7. Identification of putative promoter sequences.

Sequences to be tested as promoters were identified in two  
20 ways:

a) Sequences upstream (immediately 5' of) open reading frames in the pMH23 sequence were likely to act as promoters in the virus and as such were candidates for testing in a transient assay system.

25 b) Sequences upstream of a gene highly homologous to the 4b gene of vaccinia virus were selected by comparing the amino acids encoded by the FPV DNA with those encoded by VV 4b.

The open reading frames (ORFs) in pMH23, and the FP4b gene, were identified as follows.

30 (a) Open reading frames.

The complete sequence of the pMH23 insert (the "11.2 kb BamHI fragment") has been determined and is 11,225 nucleotides in length. This sequence is shown below (X = a nucleotide found to



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differ when sequencing from different M13 clones of FPV; asterisk = stop codon). Computer analysis of the sequence revealed the presence of several ORFs. If only ORFs of greater than 150 bases in length are considered there are nineteen complete potential genes, predicting polypeptides of between 58 and 418 amino acids. The ORFs numbered 1-12 were considered the major ORFs, either because of their size or because of their codon usage. The start and stop positions of these ORFs are shown in Table 1 below. Seven other ORFs were considered minor, either because they overlap or are contained within other potential genes, or because of their codon usage.

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1 GGATCCGACGCGGCTGCCAAGACCTTTATACCCGACTCTTGTTCTACTGGACGAACGCGG  
61 AGATTTAAAGCCATGGCTGACGTATAGTCGAGGACGCCCTCGGTAATAAATTGATTATAT  
121 TTTCAAGTTTTAAAAAATTAATTTATATGTACTCAATATCCTTATATAGAATTATTTTATC  
181 TCTTCTGATATACGTTAGGTAGATGCCGTTCAAATAATAAAATATCTGATGACGTTTTTA  
241 TGC GCGTGTTACGTTATTATAATAGATAATAGAAATAAACGTTAAAATAATAATTAATTA  
301 TCTTTTCAGTTGTTAAATATATTCTAGTTTTATAAGCGTTATTCATATATAAAAAATATA  
361 AAAACTAAATCGTATTTATTATGATGCTACGGCGGTCATTTAACAAATTTACGCGATGGA  
421 GTTCGGTTGTACGGGAACTAATAACCAGTTGGCCGTTACAGATTTACAGAAACGCGTTT  
481 TACATCTTTCAAAAAAGAACTTTTAGTTAATTTAGGAATAAGTGACTTAAATGATATAAA  
541 AAACATATGCGAGGATTCTAAAATATTCTTTCCGAAAAGAGAACGGAGCTCTTAAGTAT  
601 TAAAGATCGTAAATCTAAACAAATAGTTTTCGAAACTCCCTAAACGATGACTTGCTTAA  
661 AAAATTACACGCCTTGATCTATGATGAATTAAGTACGGTAGTAGATTCCGTTACCGTAGA  
721 GAATACCGTTACATTGATTATGTATGAAAAAGGAGATTACTTTGCCAGGCATAGAGATTT  
781 TAGTACCGTCTTTTCTAAAAACATAATATGCGTTCACCTGCTTCTATATTTGGAACAACC  
841 AGAAACGGGAGGTGAAACGGTTATATATATCGATAATAATACGTCAGTGAAATTA AAAAC  
901 AGATCATCTATTTGATAAACTATAGAACATGAAAGTATTACCGTTGAAAGCGGTAGAAA  
961 ATGCGTGGCGTTATTCGATGTCTTACTAGAAAAAAAGTTATCCGCGTCAACAAACGTAAT  
1021 AGGTAGCATAGAATACTTAGGTAAAAAAATAAATTTATATGACAGAGAAAATGATCTTCA  
1081 GTTGTGTTATTGTGATATGGTAATAGAAAGAATGACAGAAGATAAAGAATATAGCCTAGG  
1141 AATGATATCTGATAGATCAGGTAGATGTATAAAATCTCATCATAACGGTAGTATTGTTAG  
1201 ATACCGTAAAGAAGAATATGGATCTTTGATGCTCTATGTATATATAACATGAATGAAGT  
1261 GGATGAAATTTGGACTGGTGATAAGAAACATATTATATGGTCTACTATTGATAAAAAAAC  
1321 AGGAACGTCTTTTATACCTATAGATCCTGTACTTTACGAAAAGTTAAAAGCTATTTCTTC  
1381 TAAAGAGCATAAAGAATACAAAGATTTGAGAGGGTTTTGTAATAGCAGAACGGAGTATAT  
1441 TTGTTGTTCCGTATCTAAGTACTATTTGACTTACCTACAAAAACAGATTTAATACACGA  
1501 GGTGATTAATTCTATCGATTATGATACTAAGTCAGTGGGTACACCCGACTGGTATACTCT

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1561 GCCTATACAAGTTAAACAACTATCCTAGGTAATATGTCTTACGAAGAGTTATTTAATAT  
1621 AGTAAGAGGTAATATAGCTCTTGAAGAAGACAATGAATATGGCTGTGATTAACATTAATG  
1681 GTAATACTTTTCTAAAACTAATCTCAAGTATTGTTTACAAGCGACTGAAGTAATAGTTT  
1741 TAGCAAAATAATACCTTTACTGTTAGTTCTACAATCGAAATTATGCTGTAACATGAGGTA  
1801 AGGATATATTTATTAATACGTTACATCTTTCGAAAGACTTTGATCGTAGTATAATATTAT  
1861 ACATCTGCTCTACTTATTATACATAAGAAAATTTGTATTTTATTTAGTGCGCTGATAAAT  
1921 CGTGTTTAAAGTATACAACGGACGTCTATTTCCAAAAAATCTGCGCGTGTTAACGGATTA  
1981 AAATCTACATGAAAATATCTCTTAACTTTATTTCTACGTATAACAAACAACAGACTGAT  
2041 TTTATATATTACGAATAACTATTTTCTTAGGTTTTTTATATAGATGCTATACAGTGT  
2101 TACGCGTATATACAAAATACGGAAAAATAATAAACAGAAATGATTCTGGCAATATACGA  
2161 CCGCAATGCCTATATTGTTAAAAAACAGGTATCGGAAGTATCTTGTTACGCGATAACGG  
2221 TACTAGGAATACTATGCTTAATATTATTTACGATACTAGTAGTCGTAACATGCAATGGT  
2281 ATTACGCGTTTCCGTACTTTAGCAAGGTATGTCCTGATGAGTGGATAGGATATAATAGTA  
2341 AATGCTACTACTTTACTATCAATGAACTAATTGGAATGATAGCAAAAACTATGCGATG  
2401 TTATGGATTCTTCATTGATAAGGTTGATAACATAGAACTCTAAATTTCTGTGTCGCGAT  
2461 ACGGTAAGGGTAGTTACTGGATAGACATAAATCAAAATAGAAAAATTCCGGGTATTAATT  
2521 TCTCACTATATTATGAACAAGGCGTTAATGATATTTGTCTATTATTTGACACGAGTAACA  
2581 TTATCGAAATGTCTTGATATTTTACGAAAGAACGATATGTGTTAAGAAGATAGATACA  
2641 CCCATTGGTATACCGAATACATGCGTTAGATTTACTACCTCTTTTTTATACAATAGTATT  
2701 TTGTACGTTCTTGTAACAGAAAATCCGTATAGTTTATATTTTAAATCAAAGTAATAACG  
2761 AATATCTCGATGTCACGTATAAACGCAGATTCTAGATATTAAATTCTCAACGTACGTCAT  
2821 TTGCATTCCCTGAGATGATACTTTGCTATTTTATTATACCGTAGTCTATACAACCACTAC  
2881 AAAGTTAAACGAAGTAAATTATTGATTCGTTGTTATTATTTTCAGCACAGTAGTACTCGC  
2941 TATCTTCGTTTAAATCTAATAACACGCCCTTTGAAACATTTTGTGCTAGATAATAATAC  
3001 GTTATTATTACACTAACCTGTATTTCTTCTAATCTTTAAGGTGTGCTAACGATATATCAC  
3061 GGGATTAAAAGGTTATTAGTAGTCGTATAACAACATAATAATAGCACATCTGTATATTTA

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3121 TATACCTCTCGAGTACATAAAAATAATATGTTTTGATAAAACGTAAATCAATAAGTGTAT  
3181 AAGGTATTATTTCTTTTAATGAAGAAATAGGACGTAATGTCTAAATCAGATTTATATTCC  
3241 CGAAAATATTTTTCTTAGATGTATATGTTAGTTAAATTACGTGATTATATTATAAGTTAT  
3301 CTGCTTACTTTAACATTATATAGTAATTATATACTAACCGATCTTAACACTTCCGTACAA  
3361 AGAGGTATGCCCGCATCTGCGAGATATTGTGATTTTCGTATTTAGATATGTGAATATAGT  
3421 TATCTACTAACGCGACTTTCCTCCAATTTACAAAGCTCTAAGGAAAAAAAAATAAATAAT  
3481 ACTACCACGTTCTCTTTTAAGAGTTAACTATTTACTCGGAGGTATCGGTATACATACAA  
3541 TTCTATATAATTTAGTTAATCGCTTTTACGCGCATAAGTCTACGTATAATGTCTTTGTT  
3601 TAAGTAACTATCCCTGGAATATTCCTAAAAATAGCGGAATTTTTGTTTGTACGTCCGCTA  
3661 CTAGGAACATGAAAGGTACGTTTCGCTTTTACGATAGGAATTTTCTTTATTCGCTCTGTAG  
3721 TGCATAATTCGGTAACACTAGCTGCTTCAGTTCCGTATTCATCTACTTTTATCACAGATT  
3781 TTTGCCTGATATTACCTATCCTCAAAGTTTTTGATCGGATATACCTACTAATTCACCTG  
3841 ACTTGAATAGATCATTACATCCCATATGGATTAGCGCGTCTTTCAAGTCTACGTCATCTT  
3901 CTAATTCGAATTTAGGTAAATAAAGAACTATTTCTTTCAAAGTCATATCTTTTTTAGATA  
3961 TTATTTTATTGATATTCTTACCGTTATTGAGAGAATCACTACTCCTAAAAATAGAAAAAG  
4021 TATTAAAATTACGTAAACATATTAGTTTTAACATCTTTTTATTTGTTTAGTATATAAACT  
4081 TATATCGTAAAGAAATATAGTTCTCTTAATTTACGTTTATTAGGAAATAAAATAGACATA  
4141 TAGATATACACCTTAGATACTTAATTAATAATGGATAGAAACATTAATTTACCCGAAGAAG  
4201 AGCTTAAATATATAAAAGAATGTTGCGAAGTTCTTTATTTACCCAGCCGACGAGAATGG  
4261 ATATAATCGGTGTTATGAATGATAGCGATATTTCTTGGAATGAAAATCTCATCATTCTAA  
4321 TGTGGAAGATGGTAAGATTTATGTGTACGACGATGAAGCTCTATACAAAGTAGCGGATA  
4381 CTATGGAAGAGTTCTCTGAAATAGGACTTATTAATCTAGGAAATGAAGTTTATCATTGTA  
4441 GAGAGGATATAAAACCTCTCCCGAAGAGGATAGGGATAAGGATGAGTATATAATGAAGA  
4501 TAAGGGAAAAAGCCAGGCAGCTTATAGATAATTCACAAAAAGATTTTGAGGCCATTCTAG  
4561 ATTCTTTGGAGAATAAACATGTATCAATTTAGGTATATAATATAAGGTAGCAAAATACGT  
4621 ATGTCCGTGTACGCTTATGTATTTTTTTATTTGGATTAAATCGATACGCTAGAGAATAG

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4681 CGGAGTAGCTTCTGTATCCGCCGCGGTTATTTACTTTAGTAATCTATTAACTACTTTTA  
4741 TCTCTATTATTAAGTTAGTCATACCCACGAATATATATTCATAAAAACATCTTCCTCTCA  
\* K L K T L E E K L C K N  
4801 GATTTTCATCCGTAAAATTATTACTTTAATTTTGTTAACTCTTCTTTTAAACATTTATTT  
E E K L A L L Q I R I E T I Y R S Y I N  
4861 TCCTCTTTGAGAGCTAAAAGTTGATTCTAATTTGCGTTATATACCTACTATAAATATTA  
D I I K Y Q N K L Q D V I M C L E K I E  
4921 TCTATAATTTTATATTGGTTCTTAAGTTGATCTACTATCATACATAATTCTTTTATCTCT  
Q R Y K E D L E K L I S N C K I D I E S  
4981 TGGCGATATTTTTCATCGAGTTCTTTTAGAATACTATTACACTTTATATCAATTTCTGAT  
K I E K I N S D Y E E N I T K I F D S M  
5041 TTTATTTCTTTTATGTTACTATCATATTCTTCATTTATTGTTTTATGAAATCACTCATT  
(ORF5)  
V S G L A K N N R R I I M  
5101 ACACTTCCAAGAGCTTTATTATTCTACGTATTATCATTTTAAAAATCTAAATTATTGTT  
5161 TATTATATTTACATATGTTATAAACATTATTTTTAAGTATTGCCAATTAATAAATATAGT  
5221 TCATCACGATCATCTGAAGTATCTTATCATCCCGCGGCATAATTTTATATTTTAGTATAT  
5281 TTGGTTTATTACGTGCGTAGATTTAGAATCTTTATTCACACCCGATTATTGTGTTGATAG  
5341 TATATAATATTAACAATGGAGTTTTAAGCTCTACCAGAAGATATCATTAAGTATAGCG  
5401 TTCTATATGATCTAAAACATGTATATTGTACCTAGTGATAATAGCATTTTTACCATTTTC  
5461 GTTTATATTGCTAGCTCATCTATACGTAACCTTTATGGTTTATTAGCTATCTCATGTAAC  
5521 ACATATTGTTATCATCGTTTAACAGTATTATTTCTTTTAACTGATCCATTAACTTTTTT  
5581 TATGTATTAGCTCATATTCTAATTGATAAGAATCTTGTATGTAACATTTTATAAACTTTA  
5641 CTACCTTCAAAGAAAATAGAGGAGAAATCCAATGTGAAATATGTAATATAAGGTCGCGGT  
5701 GGACGTACAATTCACCTTGTTTTGCTGTCCGATACCACATTTAATACTATTCCCCTATAAT  
5761 CGTAGTAGTCATTGCATGATCTATTTATCCTGTCTAATTCATTTATTAATTCTACGGAGG  
5821 ACTCCTTACTCATCCAATCTAATATATCTCTTCCTCTAGAACTACATAACCTTGTAGCAT  
5881 TTATGTATTCATTTTCTTTCATCATAATAATTTCTATATCTTCGTAACCTAGCTTACAAA  
5941 AGTTATTATTGATCGATTCTACTTTGATTTCCATATTGAATAATTGTTATAAGCTGGAAT  
6001 ACAAATACTTAATTTTCATAATTTGTTAATAACCTAAATATTTGTATTTCTCTATAAAAA

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6061 CCACATACAAAACTATTTACATTATTATTCCAGACAATAGATTATGGTATTTTTGGGAT  
6121 CGGTACAAGCAAGTGTTATAAAGCAAGTAAATCTGGCCTCGAATTCAACATAATCACCTT  
6181 CCACAACATAACCGCTTTCTTCTTCCGAAGATTCCGACAATCGCTATGATAAAAGTATTT  
6241 ACTAGTCGTTGAAATAAAGATGTAGAATTGCCATTATATTATAATTTAGTCACTTATTT  
6301 GTTTATTTTTTTAGTACACGCTCTATCTTTCTTTACATCATAAGGCAATATTTATCATAT  
6361 ATCACGATAATCAGGATATTTATATATGTTTAATAACGGCTTTTACGTTTTATTGATTAA  
6421 GACGACACGGTAACAAAATTAATATACTTATATTGTACTACATAGTTAGCAAAATATCTA  
6481 TTAGAATACTTGTTTTGCCTATGTTTACTTCTATATTGCTATATAAGACTTATCACCTTC  
6541 AATATTTCTGTTTGTACCATATTCATGACTAGATTTTTCTATATCAAAATATATATTTAG  
6601 TTATAAAAATAATTTTATTTATAGATGTGATGTCAAGCTCTTTATTGCCTATATATTCA  
6661 AGTATGTTGTATTTTATTTATAGATGCGATGTCAAGCTCTTTATTGCCTATATATTCAA  
6721 GTATGTTGTATTTTATTTCTGTTGGGTAACCAATTCCATTTTGTTCATCACCAGTAATTT  
6781 TTTTATCTATAACTCGCATCGCTGATTCAATAGCTTCCGCTCTTTGCGATGCCGTGTCTG  
6841 CCAATTCTTTTAATAGATATTTGTAGAATATGGCATTATCATACAGACCTAATATTTTTTC  
6901 TAGAATGTCTTGCCAATATGTTCTCATCAAGATTTTTGGATGGTTTTAAACACAGGTCCA  
6961 GAATGTTGTAGGTTCTGATGCTTTCGCTGTTTATTCTCCTTAATTCAATTTTACATTTTT  
7021 CAAATACATCTTTTAAACGACTTTTGCTGTTAATGACTGTCATGTTTCTGGAAAATCCTT  
7081 TATCCGATGATATTGTATTTGTATATTGTCTTAATGCTATGTCCGCTATCAGCATATCCA  
7141 CGGATTCAGATTCTGGATTTGTATCCATATTACAGATCATCTCTAAAGTTGTGTGTTCTT  
7201 CATTATCACGGTAAACACAATGTTACTATCAGCGCTCTCTTGAGAAACATGCTTACCA  
7261 TATCTATTTTGTGTTTTGTATAGCGTAGCACATCGCTGTCACACAGGGCCTTTTGCTGA  
7321 AATAGTCAATGTTTGTCTCCGAATCTAGCAACATCCTGCATACTTCTGTGTCTCCTTTGC  
7381 TCATGGCGATGATAAGGGGAGTACATCCGTAGCAATCTTCTATGTTGGTACAGGCTCTGT  
7441 GATCTAATAGCAATTCTATACCTTTAATATCTTTTGACATAACAGCTAAATGGAGAGGCG  
7501 TAAAACGATCAGTGTTGGGCACATCAGTGTGGCTCCTCTAGCTATAAGGAGCCTCATCA  
7561 TGTCAGATTTTTACTAATTGTGGCCAAATGTAAGGGAGTGTTCCTTTCTTGTAGATAA

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7621 CATCATTTATGAACTTTCCAGAATCTAATAATTCTTCCACTTTAACACGTCCTCTCTT  
7681 CCACGGCCTCATGCAATTCAGATTCTATATCCGGATAGTTATAATCGGGATAAGTGTGT  
7741 AACTCATCAGTAATTTAATCATTTCAACATCTCTAAGTCTGACGGCCATCTTTATAGGCG  
7801 AGTATCCGTTGATAGTAAAATTCGGATTGATGTAAGAATCCAACAGGCGTCTAGCCACAT  
7861 CCAGTTCTCAAAGAGAATAGCATTGCAAAGTTCTACACGATCCATTGTATAATATAGGT  
7921 GTTCAACACCTCTCGATATATCATTATTTGTTTTTTCAATTTTATTATAAGTAGTTTGAA  
(ORF8)  
M E E G K  
7981 TGCATTTTTAAGTTTAATAAATCTTGATAAAGTATATTTAAAAAATGGAGGAGGGTAAAC  
P R R S S A V L W M L I P C G S I I I V  
8041 CGCGACGTAGTAGCGCAGTATTATGGATGTTGATTCCATGCGGAAGTATTATTATCGTGC  
L S V F V I I L S T R P P V P P D I K I  
8101 TATCTGATTTGTGATTATTTTATCCACAAGACCTCCTGTACCTCCAGATATTAAATAC  
L Y C K E G W V G Y N K N C Y F F S E E  
8161 TTTACTGTAAAGAAGGATGGGTAGGATATAATAAAACTGCTATTTTTTCTCTGAGGAAA  
K N N K S L A V E R C K D M D G H L T S  
8221 AAAATAATAAATCATTAGCTGTAGAAAGATGTAAGGATATGGACGGGCATCTGACTTCAA  
I S S K E E F K F I L R Y K G P G N H W  
8281 TTTCTAGCAAAGAAGAATTTAAATTTATCCTAAGATACAAAGGTCCGGGAAATCACTGGA  
I G I E K V D F N G T \*  
8341 TTGGAATAGAAAAAGTTGATTTTAATGGAACCTAGAAATTAGAAGATGGGTCATCTTATG  
8401 ATAATATAGTTCCTATCAAAGGAATAGGTGATTGTGCATATTTAAGCGATAGATCTATAA  
8461 TGTCGTCATTTTGTTTTTTACCGAAGAAGTGGATATGCAGAATAATACTTTTATAGAAAT  
8521 GCTAGCTAATAATGTATAATATTTTTATGAAAAATGGAAATTGATATGCATAATTATAA  
8581 CCAAAGTATGATATTGCAAGATGTCTTGATACTTTGATCATAGGTATACATGAGCAGT  
8641 TTAAATATGCAAATACAGATATAACTATTAAGATGGTGATAATAACACCGAAAGTCTTG  
8701 GAAGATGATAGTTTATCAGAATCAAGTATCCATTTTGCGAATAACAGATTCCATTTTGAT  
8761 TTGTATTATATAAAGCCTTGGGCCTTCGTAAGTATATTATATTTATTTTTATGTTTTTA  
\* E Y N N A V A P K A T  
8821 TATAATATTATTTAAACCTTTACTATTCGTAATTATTCGCTACCGCTGGTTTTTGCCGTT

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T E I Y D R I K V I I E N F S D G S L M  
8881 GTTTCTATATAGTCTCTTATTTTACTATTATTTTCATTAAATGAATCACCACCTAAGCATA  
I K S I N I R D E A H Q K R I G S I I S  
8941 ATTTTAGATATATTAATACGATCTTCTGCGTGTTGTTTTCTTATGCCGCTAATTATTGAC  
L Y R R N N E E I E A E I A A I M E D T  
9001 AAATAGCGACGATTATTTTCTCAATTTTCAGCTTCTATAGCAGCTATCATTTTCATCTGTA  
R I D E N N D I D Q E M S Y D K E L V R  
9061 CGGATATCTTCGTTATTATCTATATCCTGTTCCATAGAATAGTCTTTTCTAATACTCTT  
E A K T L L Y T S L K S Y Y S T E I E K  
9121 TCAGCTTTTGTTAGTAAATAAGTACTCAATTTACTGTAATAAGATGTTTCTATTTCTTTA  
F R N S I D K I S R D Y V E D I K S I Q  
9181 AAGCGATTACTTATATCCTTTATAGATCTATCATAAACTTCGTCTATTTTGAAATCTGA  
N Q L E Q S H D I D K E T E I N L A V C  
9241 TTCTGTAGTTCTTGGCTATGGTCTATATCTTTTTTCAGTTTCTATGTTTAGCGCTACGCAT  
R R Y Y R M L I S V R T A V Y P S I W P  
9301 CGTCTATAGTATCTCATGAGAATAGATACTCTAGTCGCTACATATGGAGAAATCCATGGA  
I V Y D L L L Q S V Y E G N I K I R T H  
9361 ATTACATAGTCCAGTAGTAATTGTGAAACGATTCTCCGTTTATTTTATTCTAGTATGC  
R I N I I P D H L K N N D K L T N L L R  
9421 CTAATATTTATTATCGGATCGTGTAACCTTGTTGTTATCTTTAATGTATTGAGCAATCTC  
R S S E L Q K W D N F D K G E L N C L R  
9481 CTAGACGATTCTAATTGCTTCCAATCATTAATAATCTTTGCCTTCTAAGTTGCATAATCTA  
T I N V F G N S R M I V V E V D L L K M  
9541 GTAATATTTACGAACCCGTTACTTCTCATAATTACTACTTCTACATCTAATAATTTTCATA  
S M F Y E N I G C I K I D T I N M K K I  
9601 GACATAAAATACTCATTAATGCCGCATATTTTATATCTGTTATATTTCATCTTCTTGATA  
(ORF10)  
T N R V E K F K M  
9661 GTATTTCTAACTTCCTTAAATTTTCATTATTATTTAGTATACTCTACAAAGTAACTAAATA  
9721 AGTTTATTTTATTTATCGGTTTTATACAAATATAAAATTTTCTATGGTGCATATATCAC  
9781 ATCCTACTATATTACTATATAAGAAATTACACATATTAATATTTGTACAATCTAGTTCGT  
9841 CTAATATTTTATCCAATAGTCCTTAGATGTATTTAATAAGCCACTATTCGTATTTATGT  
9901 TAATATTATTTCCACCGCCAAGATTATCACATACCATCATGCTATCATCCCACTTAACT  
9961 TATTTTCGGAAATAAAATAACATAAATTATCGAATTCTAACCAGTCTTTACCACACCTTA



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10021 CTAAATATCTATCTCTGTCTATATCTACTAAAATAATAACAAATAACAATATAGTGAAAG  
10081 CTATCGTTAATAGACCGGTTTCCTAGCTTTTTTACACATTTTCTTATCATATTTATATT  
10141 ACTGTTTTTTACAATTTTAAATATTATTTGTCTCATTTTGTAGTAGTAGATTTTCGTAAGA  
10201 TCATGTCATCTAATTTTGTGAGTATCATCCATCTAATTTCTATGGGTAAAGTATACCATT  
10261 TTGTATTTACTAGGTTTGCATTCATTATATTGTTTATCTCTAATAACATTTTCATATCTTT  
10321 TTGTCAACATTTTAAATATATTTTGTATTATACGAAAACAGTTGGGAAATATTGTTTTGA  
10381 TTATATTCATTTTTTCTAATAATGTATCGGACAGTCTATACACTATAGCGATGTTATCTT  
10441 CGTTAGATAATAAATCGAAAAGACTTAAATCTTGAAAAGAATTTCCGTCGTATATCTTGA  
10501 ACGTTTTCATACGTTCTATTTCTTCTTTTATAATTTTATGCAGGAACTTAAGTATTCAC  
10561 ACTTATTAATTATTTTCATATTCTTTTCCATTCCGTTAGAAATTCTAGCTTTGTAAGATA  
10621 AGTAATACAATGATACTATATAGTTAGCAAGAATAATAGCATTATTGTTAATAGTATGTA  
10681 ACATAAAGGTGTATTCCCTCATCATCTAAAGCGTTATATCAGCACCGTGGTCTATTAATA  
10741 CCAATATATTACTAAAATCATTATATCGTTCTAATATTATTCGTGTAATATATTCTACCC  
10801 ATTCTTCCTTTATATTTATATTAGCTCCTCTAGATATGATGTAATCTAATAGGTCGTCGG  
10861 TAATAAACCTAGTTTCGTATAAGGGGGATGTATTAGTTAAAACGCTTTGTTTGTTAATAT  
10921 CGGCGCCGTGGTCTAATAATACTTTTATTATTTTAAATCTAAACGGATCGTATACTTTCA  
10981 TAGCGTAATGTATAGGGTATTTACCATTGCGCCGTCTTCTGAATTAATGTCAGCGCCGT  
11041 ATTCTATAAGCAATTTTACTATTTTACTTTCTGTTCTATTAGCAGCTATATGTATAGGTT  
11101 TCAAACAATAATGTTCTAAATTAACAATAGCTCCGTATTCTAATAGCGATCTAGCTATAT  
11161 CTACACAACCTTTTTTTATAGCCTTATGTAATGGTGGTGTAAGAACCAGAAATGTTAG  
11221 GATCC

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TABLE 1Open reading frames in the fowlpox BamHI fragment in pMH23.

<u>ORF</u>	<u>Start</u>	<u>Stop</u>	No. of <u>amino</u> <u>acids</u>	<u>Size in</u> <u>kilodaltons</u>
1	416	1672	418	48.2
2	2166	2669	167	19.8
3	4054	3608*	148	16.4
4	4170	4592	140	16.5
5	5138	4821*	105	12.5
6	5974	5519*	151	17.9
7	7906	6674*	410	46.8
8	8025	8374	116	13.2
9	8632	8835	67	7.9
10	9686	8844*	280	33.0
11	10120	9689*	143	16.6
12	10705	10139*	188	22.4

\* ORFs 3, 5, 6, 7, 10, 11 and 12 are transcribed on the complementary strand to that shown above, i.e. in the reverse direction to the others.

Sequences upstream of the eleven largest major ORFs were  
 05 cloned into lacZ translational fusion vectors for the measurement  
 of promoter activity in a transient assay system.

(b) FP4b gene.

Random clones of fowlpox virus DNA were sequenced. The  
 sequence of each clone was translated on the computer into the  
 10 six possible frames and compared to a library of published

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vaccinia sequences. Several fowlpox genes with some degree of homology to vaccinia genes were detected. One gene identified in this way was a fowlpox gene highly homologous to the vaccinia 4b gene (this is referred to herein as the FP4b gene). The M13 clone containing these sequences was used to probe an EcoRI library of fowlpox virus clones (see above) and a clone containing DNA of 2.7 kilobases was detected. The clone was sequenced as described for pMH23 and found to contain the 5' end of the FP4b gene, upstream putative promoter sequences, and the 3' end of another open reading frame.

8. Assay for strength of promoter.

(a) Translational fusion vectors.

Translational fusion vectors allow potential promoter sequences, up to and including the initiation codon of the test gene, to be fused to a gene with an easily assayable product. Thus the promoter sequences under test are in exactly the same sequence context relative to the start of the ORF as in the original gene, and only the coding sequences of the gene are altered. The translational fusion vectors used in this case have the beta-galactosidase gene (lacZ) as an assayable marker and are called pNM480, pNM481 and pNM482. They are modifications of pMC1403, M.J. Casadaban et al., J. Bacteriology 143, 971-980 (1980) made by Minton, Gene 31, 269-273 (1984). The modified vectors have additional unique cloning sites available in all three reading frames.

(b) Cloning fowlpox sequences into translational fusion vectors.

Random M13 subclones generated for sequencing purposes were used to place test sequences upstream of the lacZ gene. M13 clones which started just downstream of an ATG codon and ran in an upstream direction (into the putative promoter) were

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selected. Fragments were excised from the clones, using restriction enzymes sites in the M13 polylinker, and cloned into pNM vectors cut with suitable restriction enzymes. The appropriate clone was chosen so that relatively little of the FPV gene ORF was present in the fused protein, and the appropriate vector was chosen so that the few amino acids encoded by the FPV ORF were in frame with the lacZ gene. For that reason the vectors differed by one or two nucleotides and are designated pNM 480, 481 and 482. Plasmids containing fowlpox sequences which had generated a complete lacZ gene were identified tentatively either by a blue colour on bacterial plates or by probing with radiolabelled fowlpox DNA, and definitively by sequencing across the fusion site and into the putative promoter. Figure 2 shows how all of these clones (except number 1) were cloned into the pNM vectors. (Because the only suitable M13 clone for ORF1 was in a different orientation, different restriction enzymes had to be used. The pNM 480 plasmid was cut by BamHI and HindIII, using a BamHI site between the EcoRI and HindIII sites marked, and the HindIII site was end-repaired appropriately to accommodate the BamHI - HaeIII promoter fragment excised from the M13 vector). Table 2 gives a list of the ORFs involved, the name of the M13 clone used, the pNM vector used and the number of amino acids encoded by the fowlpox ORFs (i.e. from the starting methionine codon onwards) participating in the fused products.

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TABLE 2

Translational fusion constructs of promoters (plus part of the ORF) with the lacZ gene construct.

ORF	Starting	Final	No. amino	Nucleotide
ref.	pNM vector	construct	acids of	length of 5'-
<u>ref.</u>	<u>ref.</u>	<u>vector ref.</u>	<u>ORF</u>	<u>non-coding</u>
				<u>sequence</u>
1	pNM 480	pNMGF32	20	
2	pNM 481	pNMGJ13M	7	
3	pNM 481	pNMGE23	3	
4	pNM 482	pNMGA5	13	
5	pNM 482	pNMGK4	10	189
6	pNM 480	pNMGF6	9	
7	pNM 482	pNMGB86	13	
7	pNM 480	pNMSAU4	2	
8	pNM 482	pNMGC44	14	395
10	pNM 481	pNMGF7	3	(not yet known)
11	pNM 480	pNMGL8	37	
12	pNM 482	pNMGF78	103	
FP4b	pNM 481	pNM4b30	34	283
FP4b	pNM 481	pNM4b31	21	292

(c) Testing promoters in a transient assay system.

05 Chicken embryo fibroblast cells (CEFs) seeded in 24-cell tissue culture dishes (Linbro) were infected with fowlpox virus strain HP441 when the cells were 80-90% confluent. At various times after infection DNA was introduced into the cells by a calcium phosphate transfection procedure. The system was optimised with respect to multiplicity of infection, times for

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DNA transfection and quantity of DNA for transfection, using the plasmid pMM6 which contains the vaccinia 11K promoter fused to the beta-galactosidase gene which was found to express beta-galactosidase activity in this transient assay system in  
05 FPV-infected cells. Although there was variation between individual experiments, the technique adopted, when internally controlled with pMM6 as a positive, and plasmid containing irrelevant sequences as a negative, worked consistently.

Cells in 24-well plates were infected at 1 pfu of FPV HP441  
10 per cell. Precipitates were prepared in 96-well plates by adding ingredients in the following order: pNM plasmid DNA (0.2µg-5µg) plus 1µg FPV "helper" DNA, 100µl HEPES buffered saline (pH 7.12), and finally 7µl of 2M CaCl<sub>2</sub>. The plates were tapped gently to mix the contents, then left at room temperature for 20-30 minutes  
15 until a just visible, fine precipitate developed. 24-well plates of cells to be transfected were pre-washed with HEPES-buffered saline at room temperature, then the appropriate precipitate added at 4 or 20 hours after infection of the cells. After 30 minutes at room temperature the excess precipitate was removed  
20 and 0.5ml 199 medium containing 5% CS was added. The transfected cells were reincubated as normal for a further 48 hours.

Beta-galactosidase activity was assayed as follows.

The tissue culture medium was carefully removed by aspiration, and the cells resuspended in 50µl of 0.25M TRIS-HCl  
25 pH 7.5, 5mM dithiothreitol (DTT). The resuspended cells were freeze-thawed three times then transferred to 96-well plates for assay of beta-galactosidase content. To each lysate was added 1µl of a buffer containing 60mM Na<sub>2</sub>HPO<sub>4</sub>, 40mM NaH<sub>2</sub>PO<sub>4</sub>, 10mM KCl, 1mM MgCl<sub>2</sub>, 50mM 2-mercaptoethanol and 100µl of 2mg/ml ortho-  
30 nitrophenylgalactose (ONPG) in 60mM Na<sub>2</sub>HPO<sub>4</sub>, 40mM NaH<sub>2</sub>PO<sub>4</sub>. ONPG is a colorimetric substrate for beta-galactosidase which changes from colourless to yellow. The assay was incubated for up to 2 hours at 37°C until colour developed, then 100µl of 2M Na<sub>2</sub>CO<sub>3</sub> was

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added to stop the reaction. The intensity of the yellow colour was determined by measuring the absorbance at 405nm of each well in an ELISA plate reader.

RESULTS OF PROMOTER ASSAYS.

- 05       The sequences from in front of the eleven largest major ORFs from pMH23 and from in front of the FP4b gene (see above) have been cloned into translational fusion vectors (vectors containing the lacZ gene) and their activity as promoters measured in a transient assay system. Table 2 above gives a list of these
- 10       constructs. Of the 14 FPV promoter constructs tested, five were found consistently to have promoter activity. These were the two FP4b constructs, the ORF8 (13.2K gene) promoter, the ORF5 (12.5K gene) promoter and the ORF10 (33.0K gene) promoter. All these are promoters of the invention. The remainder of the constructs
- 15       had lower levels of activity. Table 3 shows the results of three experiments. An asterisk denotes a construct containing a promoter of the invention.

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TABLE 3

Measurement of promoter strength in assay for beta-galactosidase using a colorimetric substrate (\* = according to the invention)

## Experiment A.

OPTICAL DENSITIES at 405nm

ORF ref.	Final Construct	Amount of DNA added 20 hours p.i.		
	<u>Vector ref.</u>	<u>0.2µg</u>	<u>1.0µg</u>	<u>5.0µg</u>
1	pNMGF32	0.011	0.057	0.04
2	not done			
3	pNMGE23	0.013	0.066	0.024
4	not done			
* 5	pNMGK4	0.026	0.098	0.103
6	pNMGF6	0.047	0.093	0.057
7	pNMGB86	0.031	0.079	0.027
7	pNMSAU4	0.024	0.065	0.016
* 8	pNMGC44	0.033	0.129	0.248
* 10	pNMGF7	0.027	0.071	0.138
11	pNMGL8	0.03	0.052	0.062
12	pNMGF78	0.04	0.063	0.069
* FP4b	pNM4b30	0.065	0.197	0.310
* FP4b	pNM4b30	0.057	0.203	0.260



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## Experiment B (DNA added earlier than in A)

OPTICAL DENSITIES at 405nm

ORF ref.	Final Construct	Amount of DNA added 4 hours p.i.		
	<u>Vector ref.</u>	<u>0.2µg</u>	<u>1.0µg</u>	<u>5.0µg</u>
1	pNMGF32	0.00	0.01	0.05
2	pNMGJ13M	0.03	0.00	0.02
3	pNMGE23	0.02	0.03	0.06
4	pNMGA5	0.03	0.39	0.08
* 5	pNMGK4	0.18	0.59	0.89
	6 pNMGF6	0.01	0.01	0.02
	7 pNMGB86	0.00	0.00	0.04
	7 pNMSAU4	0.03	0.04	0.03
* 8	pNMGC44	0.11	0.22	0.71
* 10	pNMGF7	0.08	0.10	0.16
	11 pNMGL8	0.06	0.05	0.04
	12 pNMGF78	0.05	0.05	0.07
* FP4b	pNM4b30	0.35	0.27	0.58
* FP4b	pNM4b31	0.28	0.32	0.44
Whole pMH23		0.01	0.02	0.02
No DNA		0.01	0.01	0.01

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## Experiment C (duplicate of B)

OPTICAL DENSITIES at 405nm

ORF ref.	Final Construct Vector ref.	Amount of DNA added 4 hours p.i.		
		<u>0.2µg</u>	<u>1.0µg</u>	<u>5.0µg</u>
1	pNMGF32	0.00	0.07	0.04
2	pNMGJ13M	0.02	0.07	0.08
3	pNMGE23	0.06	0.01	0.00
4	pNMGA5	0.05	0.00	0.09
* 5	pNMGK4	0.07	0.13	0.74
6	pNMGF6	0.05	0.05	0.02
7	pNMGB86	0.05	0.07	0.08
7	pNMSAU4	0.04	0.05	0.05
* 8	pNMGC44	0.05	0.18	0.65
10	pNMGF7	0.02	0.05	0.31
11	pNMGL8	0.03	0.06	0.09
12	pNMGF78	0.03	0.03	0.02
* FP4b	pNM4b30	0.28	0.30	1.24
* FP4b	pNM4b31	0.11	0.25	1.18
Whole pMH23		0.10	0.06	0.10
No DNA		0.03	0.04	0.04

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For experiment A the DNA was added 20 hours post infection, and for experiment B and C, which are essentially duplicates of each other, the DNA was added 4 hours post infection. It is interesting to notice that some of the promoters appear to have higher activity when added early after infection. For example at 4 hours post infection the ORF5 promoter can give higher levels of activity than the ORF8 promoter, whereas when it is added late it has lower levels. It may be that ORF5 is an early promoter which does not function well when added relatively late in infection. The ORF10 promoter, on the other hand, seems to function better when added later in infection. Both the FP4b constructs give consistently high levels.

Part of the sequences of the constructs used to test the FP4b, the ORF8 (13.2K), ORF5 (12.5K) and ORF10 (33K) promoters are shown below. Each sequence starts and finishes with DNA from the pNM vector involved, and shows how the intervening sequence is made up from fowlpox sequences plus M13 DNA. Two of the putative promoter sequences have been tested out in two separate constructs, each having different numbers of ORF amino acid coding sequence in the fused product. These are the FP4b30/FP4b31 pair and the pNMGB86/pNMSAU4 pair. In both cases the levels of promoter activity between the two different members of the pair were very similar, indicating that the length of fowlpox gene in the fused product is not critical.

```

-----><----- pNM481 sequence -----><----- M13 sequence -----
GCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGAATTCGAG
      10          20          30          40          50          60

-----><----- start of fowlpox (FP) sequence -----><-----
CTCGCCCTATTAACATTGCCTAGTAGTACTCCACTTTGGATAAGAAATCTGCATGATAAA
      70          80          90         100         110         120

-----><-----
TATATTGATATCCTACCACCTATTAAAGTACCATTATCTAATAGCAATAAGATAGATAAA
      130         140         150         160         170         180

CAAATGTTTTTTTGATGAAGTTATTACGTGGATAAATATATATCTTCAGGAAAAGGGTATT
      190         200         210         220         230         240

ATGTTACCAGATGATATAAGAGAACTCAGAGATGCTATTATTCCTTAAGTACGTCT
      250         260         270         280         290         300
                                     |-- start of FP4b gene
CTTTAGGTACTTATTTTGATACGTTACAAGTAAAAAACTATCAAATATAAATGGAATCTG
                                     MetGluSerAsp
      310         320         330         340         350         360

ATTCTAATATAGCGATTGAAGAAGTTAAATATCCTAATATTTTATTAGAACCTGTTTACT
      SerAsnIleAlaIleGluGluValLysTyrProAsnIleLeuLeuGluProValTyrTyr
      370         380         390         400         410         420

      end of FP sequence -----><----- sequence from M13mp10 --
ATAATAACCTAGAAAGTAATAGGATCTCATTACGGGGATCCTCTAGAGTCGACCTGCAGC
      AsnAsnLeuGluValIleGlySerHisLeuArgGlySerSerArgValAspLeuGlnPro
      430         440         450         460         470         480

-----><----- sequence from pNM481 (lacZ gene) -----><----- etc...
CCAAGCTTGCTCCCCTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCTGGCGTT
      LysLeuAlaProLeuAlaValValLeuGlnArgArgAspTrpGluAsnProGlyVal
      490         500         510         520         530

```

Part of the sequence of pNM4b31.

[illegible]

(lacZ) ----- etc...  
AACGTCGTGACTGGGAAACCCCTGGCGTT  
ArgArgAspTrpGluAsnProGlyVal  
490 500

[illegible]

----- sequence from pNM482 (lacZ gene) ----- etc.:  
GATCCCTGGCCGTCGTTTTACAACGTCGTGACTGGGAAACCTGGCGTT  
AspProLeuAlaValValLeuGlnArgArgAspTrpGluAsnProGlyVal  
550 560 570 580 590

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Part of the sequence of pNMGK4.

=====

----- pNM482 sequence -----><-- M13 sequence --  
 GCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGAATTCGAG  
           10          20          30          40          50          60

-----><--- start of fowlpox (FP) sequences -----  
 CTCGCCCGAATAAAGATTCTAAATCTACGCACGTAATAAACCAATATACTAAATATAA  
           70          80          90          100         110         120

-----  
 AATTATGCCGCGGGATGATAAGATACTTCAGATGATCGTGATGAACTATATTTATTAATT  
           130         140         150         160         170         180

GGCAATACTTAAAAATAATGTTTATAACATATGTAAATATAATAACAATAATTTAGATT  
           190         200         210         220         230         240

|-- start of ORF 5 gene -----><--- sequence from M13mp10 ---  
 TTTAAATGATAATACGTAGGAATAATAAAGCTCTTGGGGATCCTCTAGAGTCGACCTGC  
   MetIleIleArgArgAsnAsnLysAlaLeuGlyAspProLeuGluSerThrCys  
           250         260         270         280         290         300

-----><--- sequence from pNM482 (lacZ gene) -----  
 AGCCCAAGCTTCGATCCCCTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCTGGC  
 SerProSerPheAspProLeuAlaValValLeuGlnArgArgAspTrpGluAspProGly  
           310         320         330         340         350         360

--- etc...

GTT

Val

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Part of the sequence of pNMGF7.

=====

----- pNM481 sequence -----><-- M13 sequence -----  
 GCGCAACTGTTGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGAATTCGAG  
           10          20          30          40          50          60

-----><-- start of fowlpox (FP) sequence (exact left end unknown) ---  
 CTCGCCXX XXXXXXXXXX XXXXXXXXXX XXXXXXXCTGACAAAATTAGATGACATGAT  
           70          80          90          100         110         120

--- FP sequence continued -----  
 CTTACGAAATCTACTACTACAAAATGAGACAAATAATATTTAAAAATTGTAAAAACAGTA  
           130         140         150         160         170         180

ATATAAATATGATAAGAAAATGTGTAAAAAGCTAGGAAACGCGGTCTATTAACGATAGC  
           190         200         210         220         230         240

TTTCACTATATTGTTATTTGTTATTATTTTAGTAGATATAGACAGAGATAGATATTTAGT  
           250         260         270         280         290         300

AAGGTGTGGTAAAGACTGGTTAGAATTCGATAATTTATGTTATTTTATTTCCGAAAATAA  
           310         320         330         340         350         360

GTTAAGTTGGGATGATAGCATGATGGTATGTGATAATCTTGGCGGTGGGAATAATATTA  
           370         380         390         400         410         420

CATAAATACGAATAGTGGCTTATTAATACATCTAAGGACTATTGGATAAAAATAGTAGA  
           430         440         450         460         470         480

CGAACTAGATTGTACAAATATTAATATGTGTAATTTCTTATATAGTAATATAGTAGGATG  
           490         500         510         520         530         540

TGATATATGCACCATAGAAAAATTTTATATTTGTATAAAACCGATAAATAAAATAAATT  
           550         560         570         580         590         600

                                  |--ORF 10><-- sequence from M13mp10--  
 ATTTAGTTACTTTGTAGAGTATACTAAATAAATGAAATTTAGGGGATCCTCTAGAGTC  
                                   MetLysPheArgGlySerSerArgVal  
           610         620         630         640         650         660

-----><----- sequence from pNM481 (lacZ gene) ---  
 GACCTGCAGCCCAAGCTTGCTCCCCTGGCGTCGTTTTACAACGTCGTGACTGGGAAAAC  
 AspLeuGlnProLysLeuAlaProLeuAlaValValLeuGlnArgArgAspTrpGluAsn  
           670         680         690         700         710         720

----- etc ...  
 CCTGGCGTT  
 ProGlyVal



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INSERTION OF GENES INTO FOWLPOX VIRUS

Foreign genes are introduced into the virus by a process of homologous recombination. This process has been described in the literature in detail for vaccinia virus and an analogous  
05 procedure can be used for fowlpox virus.

1. Infection with virus and transfection of DNA.

25 cm<sup>2</sup> bottles of CEF cells at about 80% confluence are infected with about 10<sup>7</sup> pfu (about 3 pfu/cell) of an attenuated strain of fowlpox virus in 1ml of serum-free medium. The bottles  
10 are incubated at 37°C for 2 hours with occasional gentle agitation then 5ml of 199 medium (Gibco) with 5% calf serum are added and the cells are incubated for a further 2 hours at 37°C.

30 minutes before this 2 hours is up the DNA/CaPO<sub>4</sub> precipitates are prepared. 20µg of plasmid DNA, which contains a  
15 "type 1 construct" and therefore includes non-essential regions of FPV plus 2µg of fowlpox "helper" DNA are added to 1ml of HEPES buffered saline (HBS) pH 7.12 in a plastic bijou (HBS is 0.818% NaCl (w/v), 0.594% HEPES (w/v), 0.02% Na<sub>2</sub>HPO<sub>4</sub> anhydrous (w/v), adjusted to pH 7.12 with 1M NaOH). 66µl of 2M CaCl<sub>2</sub> is added  
20 slowly down the side of the bijou. This is left at room temperature for 20 to 30 minutes for a fine precipitate to form.

After the 2 hours incubation, the cells are washed twice with HBS at room temperature and the precipitate is gently added to the cells. This is left at room temperature for 40 minutes and  
26 then 5ml of 199 medium with 5% calf serum is added and the cells are incubated at 37°C for 3 to 4 hours. The medium is then changed for fresh medium.

2. Detection of recombinants.

After the virus has been allowed to grow in the cells for 3-5  
30 days (this is when a complete cytopathic effect can be seen) the cells plus supernatant are harvested and freeze thawed three times. The progeny virus is then plaqued on CEF cells at about 500-1000 plaques per 10cm petri dish and an overlay of medium

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containing 1% low gelling temperature agarose is added. The plaques are then lifted onto nitrocellulose and probed with DNA from the foreign gene which is being inserted into the fowlpox virus by the method of L. Villareal *et al.*, Science 196, 183-185  
05 (1977). Plaques which are found to light up with the probe are picked from the agarose overlay (which has been stored at 4°C), freeze-thawed three times and replaques. The plaques are then probed again with the foreign DNA to confirm that the recombinant virus has been isolated successfully from the agarose overlay.

#### 10 INFECTION OF CHICKENS WITH THE RECOMBINANT VIRUS

Twenty two chicks, 5 days old, are placed in a container (46 x 46 x 58cm.<sup>3</sup>) A spray gun is used to create a fine aerosol using 80 ml. water containing  $1.5 \times 10^8$  p.f.u. of virus grown in chicken embryo fibroblast cells. This vaccination is repeated  
15 when the chicks are 26-days old.

#### EXAMPLE 2

Promoters are signals in the viral DNA which direct transcription of RNA. Strong promoters will therefore direct transcription of greater amounts of RNA than weak promoters.  
20 This is used as a way of identifying efficient promoters. If radiolabelled viral RNA is hybridised to restriction fragments of viral DNA, immobilised on a nitrocellulose filter, particular regions of the virus containing strong promoters might be identified. For late RNA this might be expected to be difficult  
25 since late RNA transcripts are known to run well past the end of their genes, possibly into adjacent restriction fragments, hence confusing any attempts at mapping. However for early RNA it should be a useful approach. ('Early' RNA is RNA made before DNA replication and 'late' RNA is made after DNA replication; by  
30 definition. RNA made even earlier, i.e. before protein synthesis, can be referred to as 'immediate early RNA'). A convenient method of making radiolabelled RNA of the immediate

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early class is to use a in vitro system containing purified virus, deoxynucleoside triphosphates, one of which is radioactively labelled, and a suitable buffer. This has been described for vaccinia virus by S. Venkatesan & B. Moss, J. Virology 37 738-747 (1981) and it is found that the RNA produced in vitro (i.e. in a test tube) in this manner has the same pattern as that made in vivo (i.e. in tissue culture).

#### METHODS

##### Virus purification.

10 Virus was grown in chick embryo fibroblast (CEF) cells and purified as follows: Forty 75cm<sup>2</sup> flasks of CEFs were infected with  $5 \times 10^6$  pfu/flask of PP9 (a plaque-purified isolate of HP440). The flasks were incubated at 37°C for 5 days. The cells were then shaken off into the medium and then spun down at 7,000  
15 rpm for 15 minutes. The supernatant containing the virus was then centrifuged at 15,000 rpm for 30 minutes at 4°C. The virus pellets were pooled and resuspended in 40ml phosphate-buffered saline (PBS). This was layered onto a cushion of 10ml of 35% (w/v) sucrose and centrifuged at 15,000 rpm for 30 minutes. The  
20 viral pellet was then resuspended in 1ml of PBS. This was then layered onto a 20-50% (w/v) sucrose gradient and centrifuged at 15,000 rpm for 30 minutes. The two viral bands were collected, pooled, layered onto two 20-60% metrizamide gradients (about 1ml per gradient) and centrifuged at 30,000 rpm for 18-20 hours. The  
25 viral band was then collected (1ml per gradient).

In vitro synthesis of labelled RNA. (based on the method of S. Venkatesan & B. Moss, 1981 loc. cit.)

10<sup>9</sup> pfu of purified virus particles from the above procedure were used as follows to produce labelled RNA. The virus solution was  
20 made to 0.05% Nonidet P-40 (NP-40) and left on ice for 1 hour. This was then added to a solution containing 50mM Tris-HCl (pH 8.5), 10mM dithiothreitol, 5mM ATP, 1mM each of GTP and CTP, 10mM MgCl<sub>2</sub>, 100μM S-adenosylmethionine (AdoMet), and 100μCi of

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32p-labelled UTP, the total volume being 5ml. After 30 minutes at 37°C fresh AdoMet (the same amount again) was added and the reaction incubated for a further 30 minutes. The reaction was terminated by addition of EDTA to 10mM, and the tubes were placed  
05 on ice. The virus was then pelleted by centrifugation at 30,000 rpm for 30 minutes, the labelled RNA being contained in the supernatant. To the supernatant was added sodium dodecyl sulphate (SDS) to a final concentration of 0.25% and the mixture extracted with an equal volume of phenol saturated in TE (10mM  
10 TRIS-HCl, pH 7.5, 1mM EDTA). The aqueous layer was removed and extracted with diethyl ether and the RNA precipitated by addition of 1/10 volume of 3M sodium acetate and 2.5 volumes of ethanol. The RNA was spun down at 15,000 rpm for 10 minutes and the pellet resuspended in 4ml of guanidine thiocyanate solution (6M  
15 guanidine thiocyanate, 0.5% sodium N-laurylsarcosine, 5mM sodium citrate, 0.1M 2-mercaptoethanol). This was layered onto a 1ml cushion of CsCl/EDTA (5.7M CsCl, 0.1M EDTA) and centrifuged at 38,000 rpm for 18-20 hours at 18°C to pellet the RNA. The supernatant was carefully removed and discarded and the RNA  
20 pellet resuspended in 500µl of diethyl pyrocarbonate-treated water.

#### Hybridisation to DNA.

##### a) Restriction digests

An EcoRI digest of FPV DNA, and a BamHI/EcoRI digest of the  
25 11.2kb BamHI clone were separated on 0.9% agarose gels. The DNA was transferred to nitrocellulose filters by Southern blotting. Single-stranded preparations of M13 clones from the 11.2kb fragment were spotted onto nitrocellulose and baked for 2 hours at 80°C in a vacuum (1/10 of the DNA from a 1ml culture). The  
30 filters were prehybridised in 10ml of 5 x SSC (SSC is 0.15M NaCl, 0.015M Sodium-citrate) for 2 hours at 60°C. The suspension of labelled RNA being used as a probe was boiled for 3 minutes before addition to the filters. The probe and filters were incubated, with shaking, at 60°C for 18-20 hours. The filters

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were washed in 2 x SSC, 0.1% SDS at 42°C for 30 minutes, then in 0.1 x SSC, 0.1% SDS at 25°C for 30 minutes, and thereafter exposed to X-ray film.

#### RESULTS.

05       The labelled viral RNA was found to hybridise strongly to only two EcoRI fragments in the digest of FPV DNA. One was 790bp long and the other was 3830bp. (Some larger sized bands, particularly in the region of about 6,000bp, hybridised weakly). The RNA also hybridised to a 3830bp band in the EcoRI/BamHI 10 digest of the 11.2kb BamHI fragment. Labelled EcoRI FPV DNA fragments of sizes 790bp and 3830bp, purified from an agarose gel, were used to probe, by the well-known method of Grunstein & Hogness, an EcoRI library of FPV DNA fragments cloned into pUC13. Several pUC13 clones were thus identified which were also 15 probed with the labelled in vitro RNA. The resulting group of pUC13 clones proved to fall into two categories, those with viral inserts of 790bp in size and those with inserts of 3830bp in size. The 3830bp-sized clones were probed with labelled 3830bp fragment from the 11.2kb BamHI fragment (nucleotides 6162 to 9992 20 : the EcoRI sites are underlined) and were found to be the same. The 3830bp fragment includes the whole of the strongly promoted ORF8 and ORF10 genes. Also, approximately 120bp of sequence from each end of the 790bp clone have been determined (see below). Using this 790bp clone and the sequence information given below, 25 the 5'-end of this gene and thence the promoter region can readily be identified. The following is the partial sequence determined from near both ends of the 790bp fragment. (A few nucleotides from each end have not been sequenced). The numbering above 680 is approximate as the exact length of the 30 fragment is not known. N= a nucleotide not yet determined.

- 52 -

TGTCATCATA TCCACCTATA AATGTAATAT AATTAGCGCC TGATTGTGTC GATACATTAT  
 10 20 30 40 50 60

CGGGTGAAAA GTCCACCGTA ATATTGCTTT TATCGGTTGT ATTTACCACG TATAC-----  
 70 80 90 100 110

----- sequence not yet determined -----GTTCT  
 680

TTTTCATTTT TAATGTACGT TATTTTGTA TAAATGTTTAT ATAAATTACC ATACTTTANN  
 690 700 710 720 730 740

NATTATAAAT ATTGAAGTAA AAGAATAGTC TAAATTACCT AACATAGAAC ATCAT  
 750 760 770 780 790

b) M13 clones from the 11.2kb fragment.

A series of single-stranded M13 clones from the 11.2kb BamHI fragment were spotted onto nitrocellulose. Clones were chosen so that each major open reading frame (ORF) in the fragment was represented by one clone in the same orientation as the expected RNA from that ORF (i.e. unable to hybridise to the RNA) and one clone in the opposite orientation (i.e. expected to hybridise to RNA from that ORF). The clones were as follows.

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ORF	Clone reference	Nucleotide Start	No. Finish	Expected to hybridise? (+ = YES; - = NO)
1. (416-1674)	GC47 GC50	407 860	725* 545*	+ -
2. (2166-2671)	GB53 GF18	2682 2639	2887* 2581*	+ -
3. (4055-3606)	GD45 GA28	3706 3887	3918* 3627*	- +
4. (4170-4594)	GF48 GF95	4096 4481	4305* 4228*	+ -
5. (5138-4821)	GF73 GG2	5078 5041	5404* 4727*	- +
6. (5974-5519)	GE3 GF110	5604 5824	5821* 5601*	- +
7. (7906-6674)	GC59 GC61	7000 7283	7290* 7005*	- +
8. (8025-8376)	GF74 GB150	7977 8351	8238 8085*	+ -
9. (8632-8837)	MFP344 GJ24	8781 8785	8980* 8584	+ -
10. (9686-8844)	GC43 GB84	9277 9495	9499* 9230*	- +
11. (10120-9689)	GC45 GB161	9813 10107	10066* 9828	- +
12. (10705-10139)	GB64 GF21	10359 10584	10571* 10276*	- +

\* This is not the actual end of the clone, but merely the point up to which it was sequenced.

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RESULTS

Only the following clones hybridised to the in vitro RNA:

- GG2        very strongly (ORF 5 promoter)
- GC61        weakly
- 05 GJ24        very strongly (despite the fact that it is a "same  
                 orientation" clone)
- GB84        moderately strongly (ORF 10 promoter)

These results give a reasonable confirmation of the use of the RNA transcription method of identifying an immediately early strong promoter. Thus, the clones containing the ORF 5 and ORF 10 promoters hybridised strongly to the mRNA. No signal was obtained from the clone containing the ORF 8 promoter, presumably because it does not act at the immediate early stage. The strong hybridisation of GJ24 (nucleotides 8785 to 8584) is probably a result of the mRNA transcribed for the ORF 10 gene (nucleotides 9686 to 8844) running beyond the end of the gene at 8844, well into the DNA which encodes ORF 9 (8632 to 8835).

It follows that when an immediate early promoter is required, the ORF 5, ORF 10 and "790 bp" promoters appear likely to be the only good choices.



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CLAIMS

1. Fowlpox virus (FPV) promoter DNA, for promoting the transcription of a foreign gene inserted in a FPV vector, said DNA comprising the promoter of any one of the following FPV DNA genes and consisting substantially of sequence to the 5'-end of  
05 said gene which is non-coding for said gene and up to 150 nucleotides long:

(1) The FP4b gene which encodes a protein of about 657 amino acids in a sequence beginning

Met Glu Ser Asp Ser Asn Ile Ala Ile Glu  
10 Glu Val Lys Tyr Pro Asn Ile Leu Leu Glu

or a variation of such sequence;

(2) The BamHI fragment ORF8 gene encoding a protein of about 116 amino acids in a sequence beginning

Met Glu Glu Gly Lys Pro Arg Arg Ser Ser  
15 Ala Val Leu Trp Met Leu Ile Pro Cys Gly

or a variation of such sequence;

(3) The BamHI fragment ORF5 gene encoding a protein of about 105 amino acids in a sequence beginning

Met Ile Ile Arg Arg Asn Asn Lys Ala Leu  
20 Gly Ser Val Met Ser Asp Phe Ile Lys Thr

or a variation of such sequence;

(4) The BamHI fragment ORF10 gene encoding a protein of about 280 amino acids in a sequence beginning

Met Lys Phe Lys Glu Val Arg Asn Thr Ile  
25 Lys Lys Met Asn Ile Thr Asp Ile Lys Ile

or a variation of such sequence; and

(5) The gene of which the coding strand hybridises strongly to FPV RNA and is at least partly located within an approximately 790 bp DNA sequence, containing near its 5'-end  
30 the sequence:

(5') TGTCATCATA TCCACCTATA AATGTAATAT and near its  
3'-end the sequence:  
AAGAATAGTC TAAATTACCT AACATAGAAC ATCAT (3').

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2. FPV promoter DNA according to Claim 1 wherein the non-coding sequence is of length up to 100 nucleotides immediately preceding the start codon of the gene.

05 3. FPV promoter DNA according to Claim 2 wherein the non-coding sequence is of length up to 80 nucleotides immediately preceding the start codon of the gene.

4. FPV promoter DNA according to Claim 2, within any one of the following sequence of 100 nucleotides immediately preceding the start codon of the gene, as follows :-

10 FP4b (5') TATTACGTGG ATAAATATAT ATCTTCAGGA AAAGGGTATT ATGTTACCAG  
ATGATATAAG AGAACTCAGA GATGCTATTA TTCCTTAAGT AGTTACGTCT  
CTTTAGGTAC TTATTTTGAT ACGTTACAAG TAAAAAACTA TCAAATATAA  
(3')

ORF8 (5') AGAATAGCAT TGCAAAGTTC TACACGATCC ATTGTATAAT ATAGGTGTTC  
15 AACACCTCTC GATATATCAT TATTTGTTTT TTCAATTTTA TTATAAGTAG  
TTTGAATGCA TTTTAAAGTT TAATAAATCT TGATAAAGTA TATTTAAAAA  
(3')

ORF5 (5') TAAACCAAAT ATACTAAAAT ATAAAATTAT GCCGCGGGAT GATAAGATAC  
TTCAGATGAT CGTGATGAAC TATATTTATT AATTGGCAAT ACTTAAAAAT  
20 AATGTTTATA ACATATGTAA ATATAATAAA CAATAATTTA GATTTTAAAA  
(3')

ORF10 (5') ACTAGATTGT ACAAATATTA ATATGTGTAA TTTCTTATAT AGTAATATAG  
TAGGATGTGA TATATGCACC ATAGAAAAAT TTTATATTTG TATAAAACCG  
ATAAATAAAA TAACTTATT TAGTTACTTT GTAGAGTATA CTAAATAATA  
25 (3')

or within a variation of such sequence.

5. A recombination vector comprising a cloning vector containing, as an insert, a non-essential region (NER) sequence of FPV, said NER being interrupted by DNA comprising (a) promoter  
30 DNA according to Claim 1, 2, 3 or 4 followed by (b) a foreign gene transcribable by the promoter.

6. A recombination vector comprising a cloning vector containing, as an insert, in order:

(1) a first homologously recombinable sequence of the  
35 fowlpox virus (FPV) genome,

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- (2) a sequence within a first portion of a non-essential region (NER) of the FPV genome,
- (3) FPV promoter DNA according to Claim 1, 2, 3 or 4.
- (4) a foreign gene transcribably downstream of the promoter (whereby when the fowlpox virus RNA polymerase binds to the promoter it will transcribe the foreign gene into mRNA),
- (5) a sequence within a second portion of the same NER of the FPV genome, the first and second sequences being in the same relative orientation as are the first and second portions of the NER within the FPV genome, and
- (6) a second homologously recombinable sequence of the FPV genome, said sequences (1) and (6) flanking the NER in the FPV genome and being in the same relative orientation in the recombination vector as they are within the FPV genome.
7. A DNA cassette which comprises a FPV promoter according to Claim 1, 2, 3 or 4, transcribably linked to a foreign gene.
8. A recombinant cloning vector containing a DNA cassette according to Claim 7.
9. A recombinant fowlpox virus (FPV) which is the product of homologous recombination of a parent FPV with the insert DNA of a recombination vector according to Claim 5 or 6.
10. An in vitro culture of animal cells infected with a virus claimed in Claim 9.
11. A culture according to Claim 10 wherein the animal cells are chicken cells.
12. A method of vaccinating a responsive animal, which comprises inoculating it with a recombinant FPV as defined in Claim 9.
13. A method according to Claim 12 wherein the animal is a chicken.

PCT

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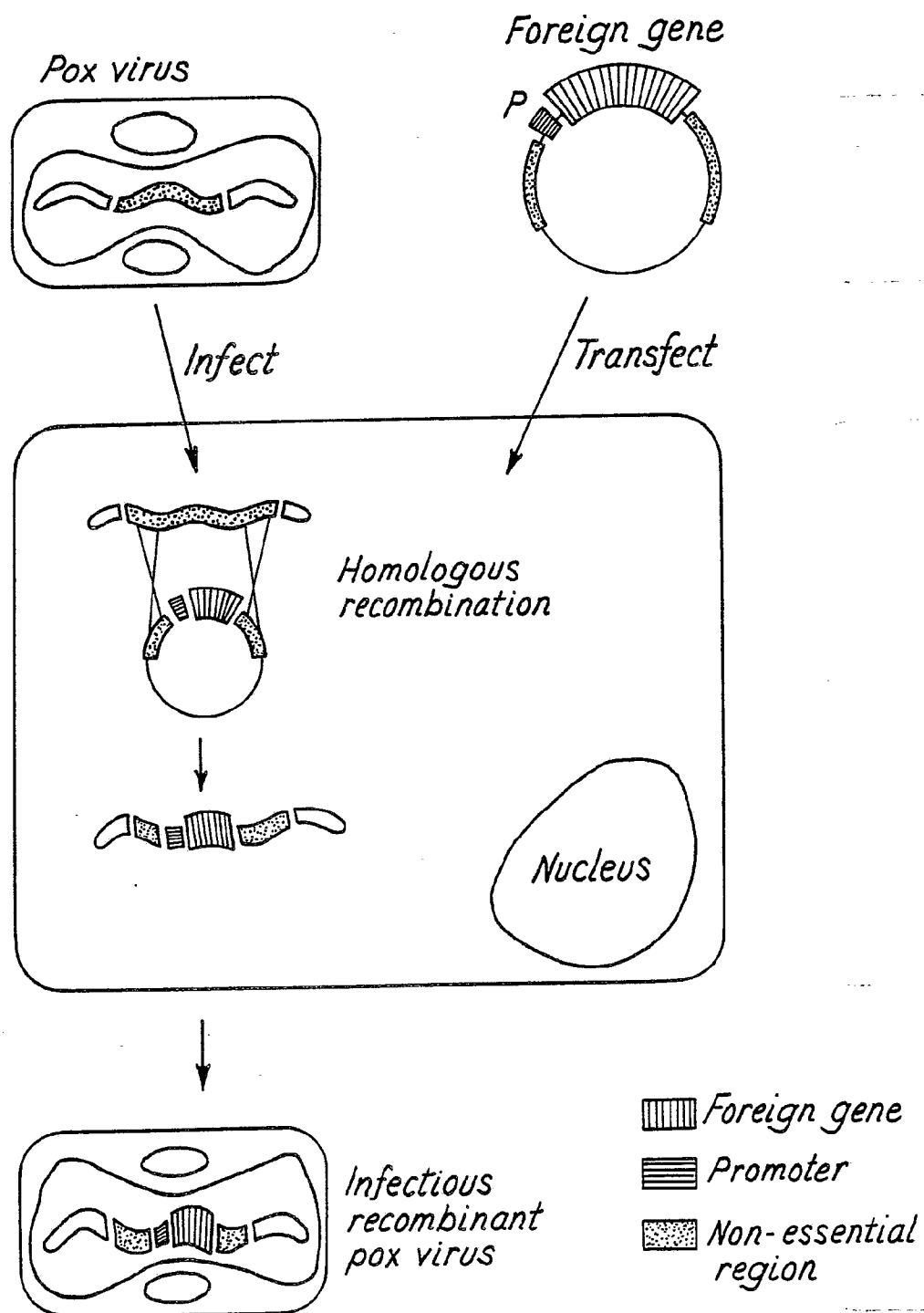


Fig. 1<sup>c</sup>

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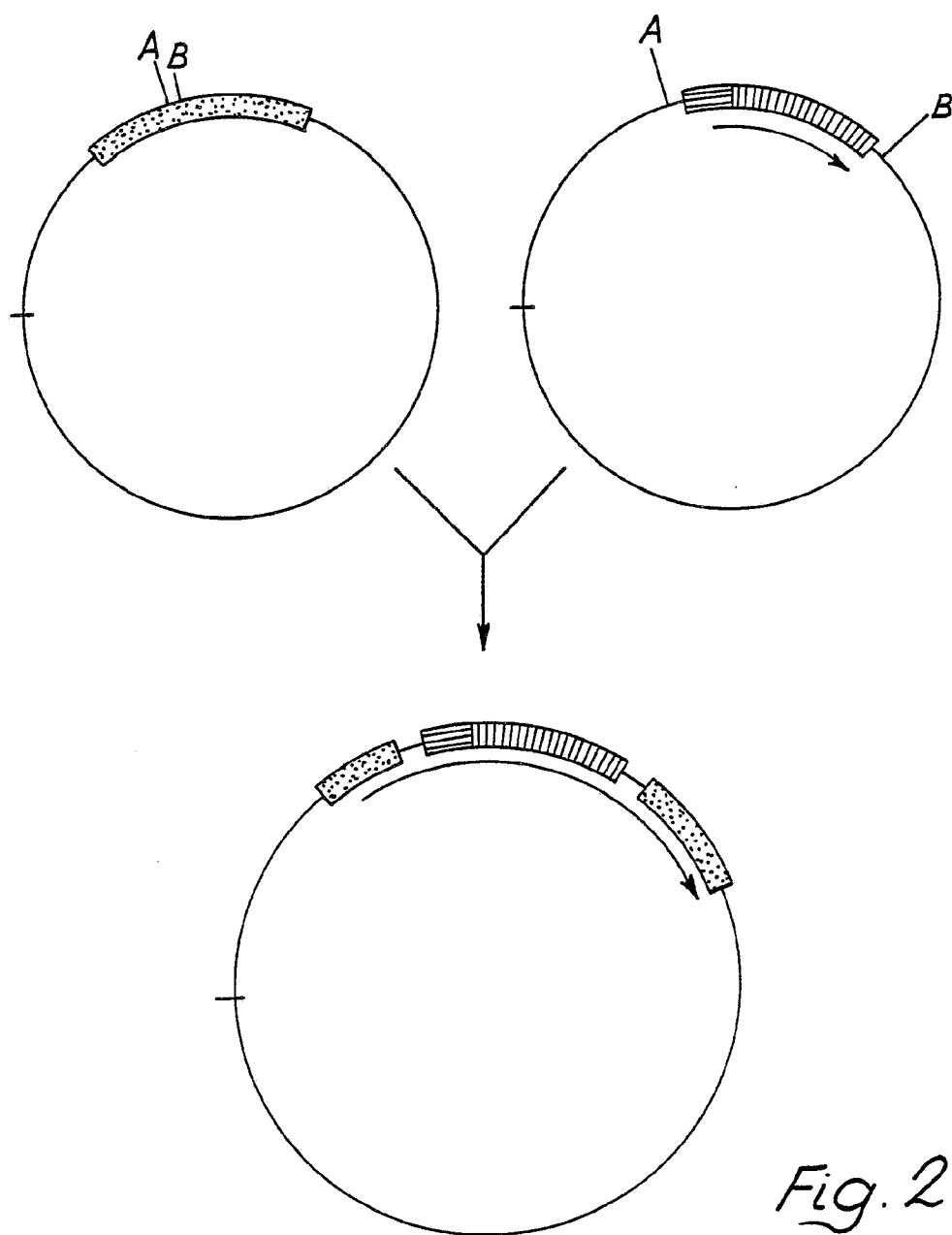






Fig. 2

-  *Foreign gene*
-  *Promoter*
-  *Non-essential region*
-  *Plasmid DNA*

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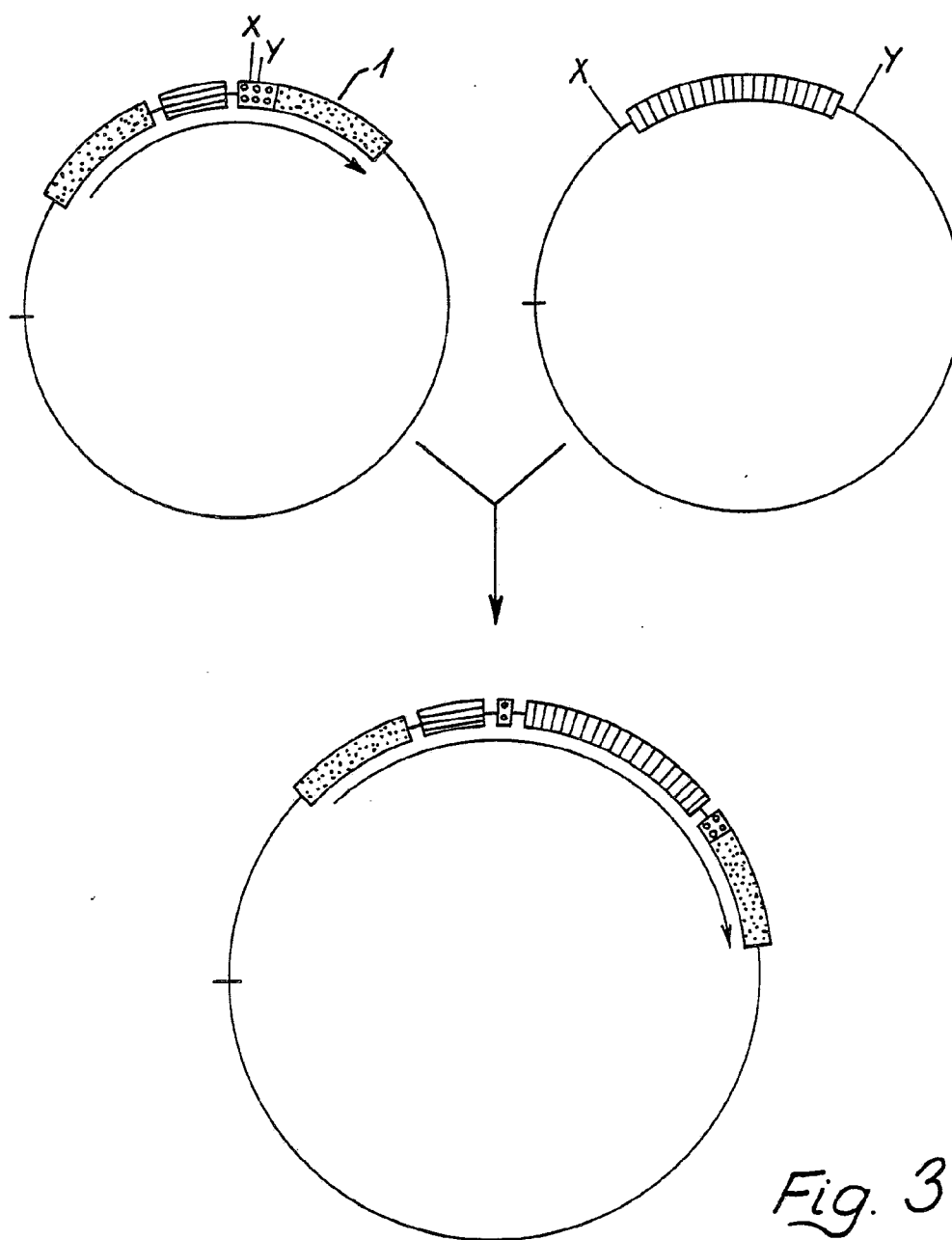





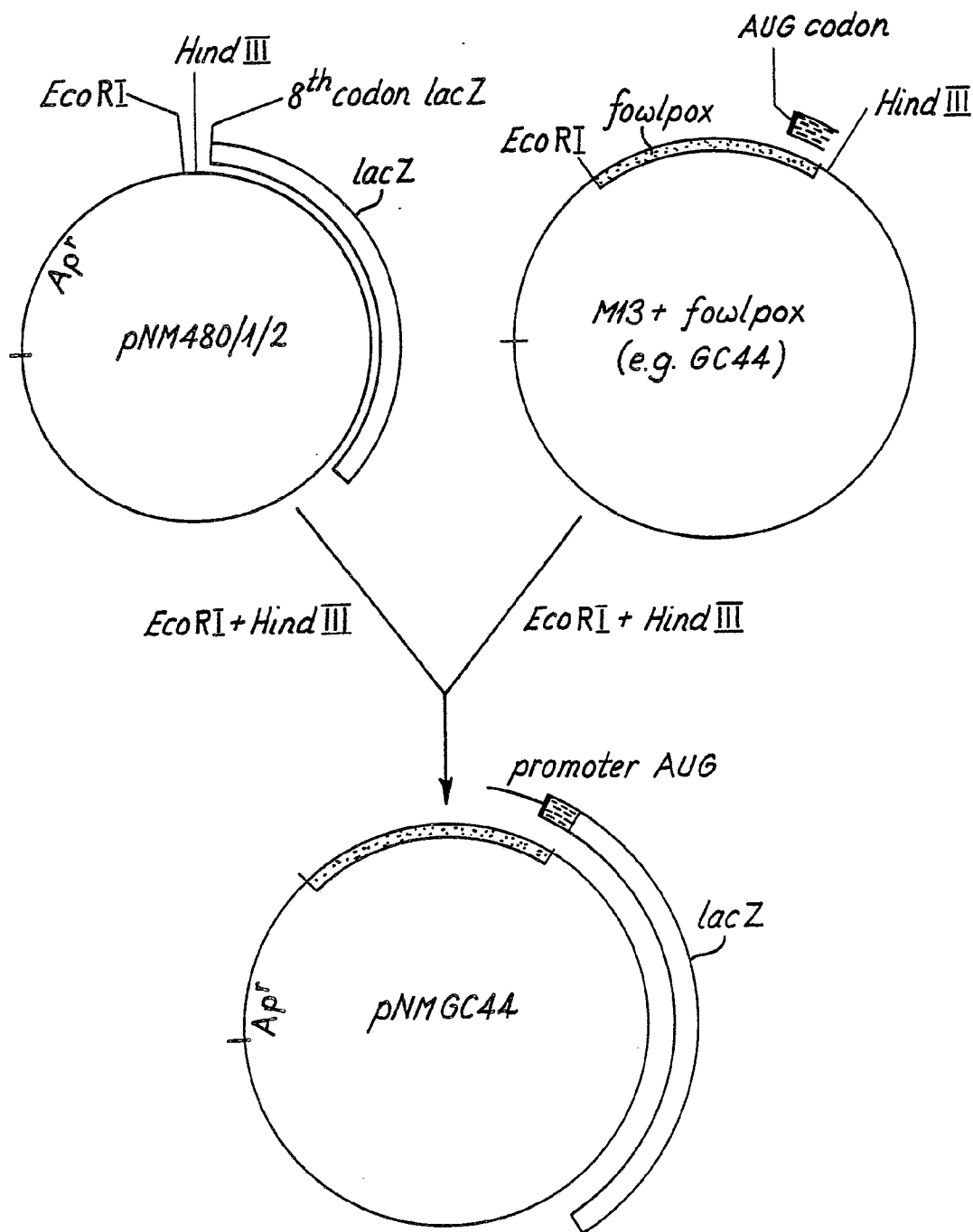


Fig. 3

-  Foreign gene
-  Promoter
-  Non-essential region
-  Multiple cloning site
-  Plasmid DNA

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*Selected on Xgal amp. plates*

*Fig. 4*

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 88/00922

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC <sup>4</sup> : C 12 N 15/00; C 12 N 7/00; C 12 N 5/00														
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched <sup>7</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 30%; text-align: left; border-bottom: 1px solid black;">Classification System</th> <th style="text-align: left; border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="border-right: 1px solid black; padding: 5px;">IPC<sup>4</sup></td> <td style="padding: 5px;">C 12 N; C 12 P</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched <sup>8</sup></div>			Classification System	Classification Symbols	IPC <sup>4</sup>	C 12 N; C 12 P								
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IPC <sup>4</sup>	C 12 N; C 12 P													
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; text-align: left; border-bottom: 1px solid black;">Category <sup>10</sup></th> <th style="width: 70%; text-align: left; border-bottom: 1px solid black;">Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 20%; text-align: left; border-bottom: 1px solid black;">Relevant to Claim No. <sup>13</sup></th> </tr> <tr> <td style="border-right: 1px solid black; vertical-align: top; padding: 5px;">Y</td> <td style="border-right: 1px solid black; vertical-align: top; padding: 5px;">WO, A, 86/00528 (GENEX CORP.) 30 January 1986, see pages 24-27 --</td> <td style="vertical-align: top; padding: 5px;">1-13</td> </tr> <tr> <td style="border-right: 1px solid black; vertical-align: top; padding: 5px;">Y</td> <td style="border-right: 1px solid black; vertical-align: top; padding: 5px;">The Journal of Virology, vol. 56, no. 3, December 1985 J. Rosel et al.: "Transcriptional and translational mapping and nucleotide sequence analysis of a vaccinia virus gene encoding the precursor of the major core polypeptide 4b", pages 830-838, see the whole document, in particular figure 5 (cited in the application) --</td> <td style="vertical-align: top; padding: 5px;">1-13</td> </tr> <tr> <td style="border-right: 1px solid black; vertical-align: top; padding: 5px;">A</td> <td style="border-right: 1px solid black; vertical-align: top; padding: 5px;">Israel. Journal of Veterinary Medicine, vol. 42, no. 2, 1986 M.M. Binns et al.: "Prospects for a novel genetically engineered vaccine against infectious bronchitis", pages 124-127, see page 126, last paragraph; page 127, in particular figure 1 (cited in the application) --</td> <td style="vertical-align: top; padding: 5px;">1-13</td> </tr> </table>			Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	Y	WO, A, 86/00528 (GENEX CORP.) 30 January 1986, see pages 24-27 --	1-13	Y	The Journal of Virology, vol. 56, no. 3, December 1985 J. Rosel et al.: "Transcriptional and translational mapping and nucleotide sequence analysis of a vaccinia virus gene encoding the precursor of the major core polypeptide 4b", pages 830-838, see the whole document, in particular figure 5 (cited in the application) --	1-13	A	Israel. Journal of Veterinary Medicine, vol. 42, no. 2, 1986 M.M. Binns et al.: "Prospects for a novel genetically engineered vaccine against infectious bronchitis", pages 124-127, see page 126, last paragraph; page 127, in particular figure 1 (cited in the application) --	1-13
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"G" document member of the same patent family</p> </div> </div>														
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-right: 1px solid black; padding: 5px;">           Date of the Actual Completion of the International Search             8th February 1989         </td> <td style="width: 50%; padding: 5px;">           Date of Mailing of this International Search Report             1 MAR 1989         </td> </tr> <tr> <td style="border-right: 1px solid black; padding: 5px;">           International Searching Authority             EUROPEAN PATENT OFFICE         </td> <td style="padding: 5px;">           Signature of Authorized Officer             P.C.G. VAN DER PUTTEN         </td> </tr> </table>			Date of the Actual Completion of the International Search  8th February 1989	Date of Mailing of this International Search Report  1 MAR 1989	International Searching Authority  EUROPEAN PATENT OFFICE	Signature of Authorized Officer P.C.G. VAN DER PUTTEN								
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International Searching Authority  EUROPEAN PATENT OFFICE	Signature of Authorized Officer P.C.G. VAN DER PUTTEN													



## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A,P WO, A, 88/02022 (COMMONWEALTH SCIENTIFIC  
AND INDUSTRIAL RESEARCH ORGANISATION)  
24 March 1988,  
see the whole document

1-13

A,P EP, A, 0284416 (NIPPON ZEON CO. LTD)  
28 September 1988,  
see the whole document

1-13

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>1</sup>

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers xx because they relate to subject matter not required to be searched by this Authority, namely:

xx Claims 12 and 13

See PCT Rule 39.1(iv)

Methods for treatment of the human or animal body by means  
of surgery or therapy, as well as diagnostic methods.

2. ☐ Claim numbers..... because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>2</sup>

This International Searching Authority found multiple inventions in this International application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

GB 8800922

SA 25064

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 22/02/89. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8600528	30-01-86	EP-A- 0190254 JP-T- 61502586	13-08-86 13-11-86
WO-A- 8802022	24-03-88	AU-A- 8075787	07-04-88
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